ANTI-PROLIFERATIVE PROPERTY OF PORCUPINE BEZOAR EXTRACT ON MELANOMA (A375)

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ABSTRACT

Porcupine bezoar (PB) has been previously reported to possess various medicinal properties. However, its potential as an anticancer agent against human cancers is still poorly studied and understood. Hence, in this study, porcupine bezoar (PB) aqueous extract was evaluated for its potential as a safe anticancer agent. Initially PB was infused in water for 18 h to get PB aqueous extract which was preliminary tested for total phenolic content (TPC), total flavonoid content (TFC), DPPH activity in order to confirm the presence of bioactive agents as antioxidants in PB. Later, PB aqueous extract was subjected to A375 (Melanoma) ($IC_{50,}$ cell

proliferation assay) and HGF-1 (normal cell, for cytotoxicity assay) to evaluate its anticancer potential. PB aqueous extract showed 11.68 µg/mL ± 0.67 TPC value expressed as gallic acid equivalents while TFC was not detected confirming the absence of flavonoids in PB aqueous extract. IC_{50} for DPPH was found to be 0.79 µg/mL ± 0.07. The IC_{50} result for A375 was found to be 10.1 µg/mL ± 0.17. PB aqueous extract showed significant anti-proliferation pattern at 24, 48 and 72 hours exposures. The data from this study suggest the potential alternative use of PB in developing natural antioxidants and anti-proliferative agents for improving human health.

Keywords : Porcupine bezoar, TPC, TFC, DPPH, cytotoxicity.

INTRODUCTION

Cancer remains one of the major health concerns of the 21st century. It has been reported to be the second leading cause of death worldwide (Park et al. 2008; Hashim et al., 2016; Siegel et al., 2016). Several research studies have reported that cancer is expected to grow worldwide, especially in underdeveloped countries which contribute to 82% of the world's population (S. Park et al., 2008; American Cancer Society, 2014; Siegel et al., 2016). In addition, the occurrence of cancer is becoming an enormous burden when available cancer therapies, such as surgery, chemotherapy and radiotherapy, are showing defective prognosis and various side effects (De Moor et al., 2013; Siegel et al., 2013; American Cancer Society, 2014). Therefore, the exploration of anti-cancer agent with minimal toxicity and highly specific becomes urgent to avert increasing cancer cases throughout the world every year. Researchers have highlighted in their study that a new definition of effective anticancer agent should be nontoxic and effective at lower doses (Amin, Gali-Muhtasib, Ocker, & Schneider-Stock, 2009; Asmaa et al., 2014).

Thus in this study, porcupine bezoar (PB) was evaluated to explore its ability as a safe anticancer agent. PB is a type of stone-like substance found in a porcupine's body. PB is a mass of indigestible foreign material which could be undigested organic or inorganic material that accumulate and conglomerate in the gastrointestinal tract, most commonly in the stomach. Although they are most commonly found in the stomach but it can occur anywhere from the esophagus to the rectum (Duffin, 2013; Sanders, 2004). PB looks like elongated acorn or unripe date, with a peel like an onion, and some- thing like green grass of blackish to green hue in the center (Barroso, 2014). It has also been mentioned that PB stone is probably the most exquisite animal medicine that has come from the east due to

its outstanding therapeutic and medicinal properties (Barroso, 2014). Moreover, PB is believed to be prince of antidote and alexipharmic due to its ability in treating deadly diseases/conditions (Barroso, 2014; Duffin, 2013).

Old medical manuscripts across the globe have explained different pharmacological uses of PB in the treatment of various ailments (Barroso, 2014; Duffin, 2013). Even, among natural products in Malaysia, PB is considered to have a great potential as chemopreventive agent despite the fact that no scientific study has been carried out so far to explore its anticancer potential. Therefore, this preliminary research study focuses on investigating porcupine bezoar's extract in inhibiting cell proliferation of cancer cells with minimal toxicity to normal human cells.

METHODOLOGY

Materials

An official permission to use the PB in this research was obtained from the Department of Wildlife and National Parks, Semenanjung Malaysia (PERHILITAN) – JPHL&TN(IP):100-34/1.24 Jld 8. The cell line A375 (Melanoma) and HGF-1 (Human gingival fibroblast) were purchased from American Type Cell Collection (ATCC), USA. The antibiotic mixture viz. Penicillin-Streptomycin (Gibco, US), fetal bovine serum (Gibco, US) Dulbecco's modified Eagle's medium (Gibco, US), phosphate buffered saline (PBS) (Gibco, US), ethanol (Honeywell, US), dimethyl sulfoxide (DMSO) (Sigma-aldrich, US), TrypLE (Thermo fisher, US) and Trypan blue (Gibco, US) were used.

Cell lines and culture

A375 and HGF-1 cells were grown in Dulbecco's modified eagle medium (DMEM) with 10 % (v/v) fetal bovine serum and 1% (v/v) of Penicillin-Streptomycin mixture. Cells were cultured in humidified atmosphere of 5% CO_2 at 37°C. Cells were sub-cultured and used for treatment once reached >80% confluence.

Porcupine bezoar extract preparation

PB extract was prepared into stock solution of 4.0 mg/mL. PB powder was extracted in sterile deionized water by stirring for 18 h using magnetic stirrer

before filtration using 22 µ pore filter. The stock solution was further diluted with complete growth media immediately prior used.

Determination of total phenolic content

Total phenolic content of the aqueous extract was quantified using modified Folin–Ciocalteu's method adapted to the 96-well plate assay (Sulaiman et al., 2011; Ahmed et al., 2015). 50 µL of 25 % (w/v) Folin–Ciocalteu's reagent and 75 % (w/v) sodium carbonate were mixed with 10 µL of sample from stock solution. The resultant mixture was further incubated for 45 min at room temperature and absorbance was measured using Tecan microplate reader (Infinite® 200 PRO) at 765 nm against blank. All assays were carried out at least in triplicate. A standard curve was plotted using gallic acid (0.1–5000 µg/mL). The results were expressed as microgram gallic acid equivalent per gram dry weight basis of fresh sample (mg GAE/g dw basis).

Determination of total flavonoid content

A modified method of Ahmed et al. (2015) and Umar et al. (2010) was followed using 96-well plate. 90 µL of 2% (w/v) aluminium chloride was added to 10 µL of sample. The resultant mixture was directly incubated for 10 min at room temperature and absorbance was measured using microplate reader (Tecan Infinite®200 PRO) at 415 nm against blank. Quercetin (0.1–5000 µg/mL) was used as a reference to produce a standard curve. All tests were performed in triplicate. The data were expressed as microgram quercetin equivalent per gram dry weight basis of fresh sample (mg QE/g dw basis).

Determination of antioxidant activity: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The modified method of Sulaiman et al. (2011) and Ahmed et al. (2015) was carried out using 96 wells microplates. In brief, samples were analysed with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as follows: 80 µL of DPPH (0.2 mmol) in methanol was put in the microtiter plate, followed by 20 µL of standards or serially diluted different concentrations of extract (0.1–5000 µg/mL) were added, while for blank, only 20 µL of extraction solvent was added to the DPPH solution. The plate was immediately placed in Tecan microplate reader (Infinite® 200 PRO), and their absorbance was recorded at 515 nm after 15 min. Ascorbic acid was used as standards and dissolved in methanol. The decreased absorbance of the DPPH

solution at 515 nm indicates an increase of the DPPH radical scavenging activity. The percentage of DPPH inhibition was used to calculate DPPH scavenging effect, using below equation:

$$\frac{A\,c - (As - Ab}{Ac} \times 100$$

Where Ac is the absorbance of control reaction (containing all reagents except the extract/standard), as is the absorbance of solution with the sample extract or standard and Ab is the blank containing extract/standard with solvent. The amount of antioxidant necessary to decrease the initial DPPH absorbance by 50% (EC_{50}) was used to define the antiradical activity of various extracts. The EC_{50} value for each extract was graphically determined by plotting the DPPH scavenging percentage as a function of extract concentration.

Inhibition concentration (IC_{50}) determination on A375

IC_{50} of PB aqueous extract on cells were determined by MTT assay. Cells were seeded at 5.0 X 10^4 cells in each of 24-well plate in 1 mL of complete growth media for attachment overnight. Prior to treatment with range concentration (5.0-160.0 µg/ mL) of PB extract, cells were washed with PBS and fresh media was added. After 72 h of the treatment, the medium was aspirated and the cells were exposed to MTT solution prepared at 5 mg/mL in sterile PBS before incubation at 37 °C for 3 h. The water insoluble formazan salt was solubilized with 1 mL of DMSO/well. Absorbance was measured at primary wavelength of 570 nm and reference wavelength of 620 nm, respectively. IC_{50} was determined using Graph pad prism version 6.0.

Cell proliferation assay

Cells were seeded at 5 X 10^4 cells/mL in 6 well plates. After overnight incubation, cells were washed, PB extract (IC_{50}) was added for treated cells. Untreated cells were grown in complete growth media as negative control. Cells were incubated in 5% CO_2 at 37 ^oC for 24, 48 and 72 h. Cells viability was analyzed using trypan blue dye exclusion method. Cells viability was measured at 24, 48 and 72 h to observe growth pattern of treated and untreated cells. Results were analyzed using Graph pad prism software 6.0. Differences were compared by multiple t test

followed by Sidak-Benferroni correction. Data values were expressed as means ± SD. P values were considered to be statistically significant when *p<0.001.

Cytotoxicity test on normal cells

Approximately 5 X 10^4 of normal Human Gingival Fibroblast (HGF-1) were seeded in 6 well plates. After 24 h of incubation, cells were washed, fresh media and serial concentrations of porcupine bezoar (10, 25, 50, 75, 100 and 160 µg/mL) were added prior incubation for 72 h. Cell morphology of treated and untreated cells were examined using inverted phase contrast microscope before number of viable cells were counted using haemocytometer under inverted microscope using Trypan blue dye exclusion method. The viability of treated and untreated cells was compared.

RESULTS

Total phenolic content

Determination of total phenol and total flavonoid contents are both colorimetric assays to quantify the presence of phenolic compounds and flavonoids in the sample. The reaction between the Folin-Ciocalteu reagent and phenolic compounds results in the formation of a blue color complex that absorbs radiation and allows quantification. The total phenolic content in the aqueous extract of PB was found to be 11.68 µg/mL ± 0.67.

Total flavonoid content

The total flavonoid assay involves reaction between aluminium chloride and the flavonoid content in the sample to give a complex formation (yellow colour). The aqueous extract of porcupine bezoar gave a negative result (-22.8224 µg/mL ± 3.81) for the flavonoid content indicating the absence of flavonoid in PB aqueous extract.

Radical scavenging assay

Among the several methods commonly used to investigate the antioxidant capacity/potential of natural products, the free radical reagent viz. 2,2-diphenyl-1-picryl-hydrazyl ($DPPH^{\cdot}$) was chosen because it is an easy, precise, and accurate method (Alves et al., 2010). DPPH is a free radical, which is stable at room temperature and produces a violet solution in alcohol. It is reduced in the presence of an antioxidant molecule. The evaluation of the antioxidant activity of porcupine

bezoar aqueous extract was determined based on the scavenging activity against the free radical 2, 2-diphenyl-1-picryl-hydrazyl ($DPPH^{\cdot}$) through the IC_{50} parameter, which represents the concentration of the material in question necessary to inhibit 50% of free radicals. The IC_{50} of PB aqueous extract was calculated as 0.79 µg/mL ± 0.07.

Table 1 Results TPC, TFC, DPPH of PB

Test	TPC (µg/mL)	TFC (µg/ml)	DPPH (µg/mL)
Result	11.68 ± 0.67	-22.82 ± 3.81	0.79 ± 0.07

Inhibition Concentration of PB on A375

Anticancer agents have the common ability in inhibiting cell progression. As far as our literature review could ascertain, no specific information was found on specific dosages of porcupine bezoar used previously as they just soaked in water for hours (Duffin, 2013; Barroso, 2014). Thus, in preliminary screening, PB aqueous extract was observed for its ability to inhibit cancer cells by using serial concentration of PB extracts. Table II shows the IC_{50} of PB extracts on A375 cells.

Table 2 IC50 of PB on A375

Cells	µg/mL
IC_{50}	10.1 ± 0.17

Cell proliferation assay

Results from IC_{50} determination for A375 showed that PB aqueous extract exhibit a dose-dependent pattern. Hence, to verify the effect of the PB aqueous extract on cell growth inhibition pattern, the cell proliferation assay was conducted to investigate the anti-proliferative activity of PB aqueous extract on A375. Cell viability for analyzing growth pattern was assessed by trypan blue exclusion assay. Figure 1 a demonstrates significant number of reduced viable cells compared to untreated cells in all incubation hour of exposure with PB aqueous extract. As shown in Figure 1 a and Table III, A375 shows increasing pattern in

reducing cell proliferation from 24 hour to 72 hour after exposed to PB aqueous extract. The inhibition were increasing from 24 to 72 hours by 13.6% in 24 hours, 22.2% in 48 hours and 45.8 % in 72 hours. It can be deduced that upon treatment with concentration at IC_{50} of PB extracts on A375, PB extracts exerts cytotoxic and cytostatic effect in dose and time dependent manner.

Cytotoxicity test

Serial concentrations of PB aqueous extract were used to test on normal human gingival fibroblast (HGF-1) for toxicity testing. Results in Figure 1b, showed in all incubation hours at 24, 48 and 72 hours, no sign of toxicity occur referring to the percentage difference of cells treated with PB aqueous extract and untreated even at the highest concentration (150 µg/mL). The result demonstrates that PB aqueous extract has high potential of anticancer agent.

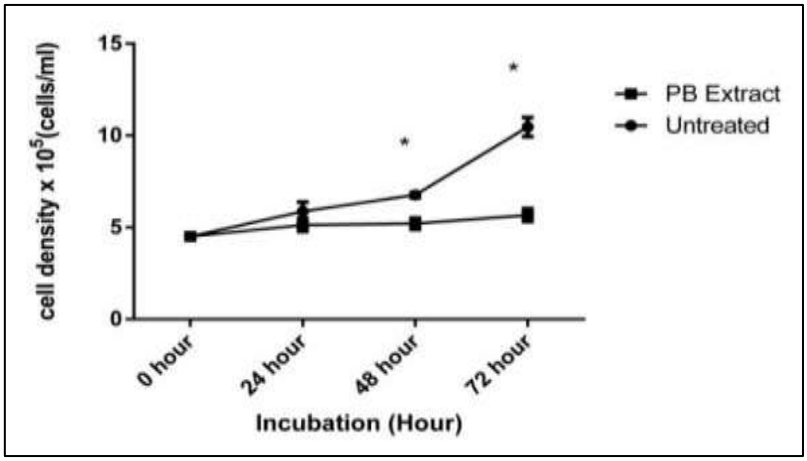

Figure 1 (a) Anti proliferative effect of PB extract on A375 cells

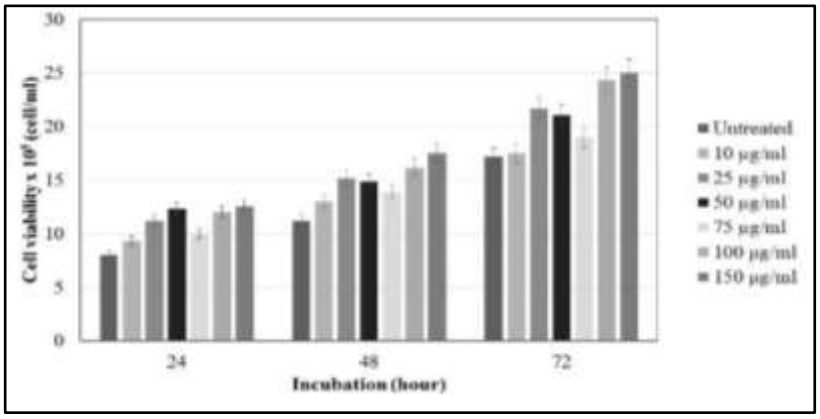

Figure 1 (b) Cytotoxicity testing on HGF-1 cells

Figure 1 *Anti proliferation (a) and cytotoxicity (b) studies of PB aqueous extract*

Table 3 Percentage differences (%) between cells treated IC50 of PB aqueous extract and untreated cells

	Time Duration (hour)		
Cells	**24**	**48**	**72**
A375	13.6	22.2	45.8

DISCUSSION

Nowadays, growing interests have focused on the efforts to control the incidence of cancers by developing effective strategies to manage cancer patients. Other than current treatment, numerous studies have successfully acknowledged remarkable beneficial health effects of natural sources which contribute as a source of effective anticancer agents (Dorai & Aggarwal, 2004). A variety of natural dietary agents have been reported to have potent anticancer activity, low toxicity and few adverse side effects (Aggarwal & Shishodia, 2006).

Based on the total phenol and flavonoid contents, the result showed that aqueous extract of porcupine bezoar contains a small amount of phenolic compounds, however, devoid of flavonoids. Phenolic compounds are considered very important bioactive agents because of their scavenging ability on free radicals due to their phenolic hydroxyl groups (Stankovic, 2011). However, the DPPH assay did not exhibit 50% of inhibition for PB aqueous extract. Therefore, the compounds that are responsible for the effect on the cancer cells could be other kinds of bioactive compounds along with phenolic compounds which were found to be present in small amount in PB aqueous extract. Moreover, flavonoids which are also classified under the category of polyphenols were found to be absent in PB aqueous extract further confirming and supporting the fact that the different

mechanism of action could be involved for mentioned effect rather than radical scavenging mechanism (Umar et al., 2010).

With respect to the determination of IC_{50} value, it is important to note that the inhibitory effect exerted on the A375 cell line was found to be dependent on the type of extract used. Higher IC_{50} was found in this study, this may be due to direct usage of crude extract from water. Lower IC_{50} values can be attained by using specific bioactive compounds from the special extraction process. Moreover, it is important to highlight here that this study is the first report on the IC_{50} of porcupine bezoar using water extract on A375 cell.

The antiproliferative effect of PB aqueous extract was evaluated by prolonging the incubation time (until 72 hours) in order to investigate whether PB aqueous extract have the potentiality to either inhibit, suppress or increase the growth of the A375 cell. The results which are shown in Figure 1(a) clearly reveal that PB aqueous extract was able to inhibit cell growth in A375 cell significantly in a dose dependent manner. Previous study on *Curcumin* reported that a compound which was able to inhibit the proliferation of tumour cells have potential as an anticancer agent (Anto , 2002). In addition, another cytotoxicity study of paxcitaxel on eight different types of cell lines, demonstrated that each cell exerted a different level by inhibiting cell proliferation and some cells, even showed signs of survival for long exposure (Liebmann et al., 1993).

It is crucial to identify whether the obtained IC_{50} can cause toxicity towards normal cell lines before further test can be made. This is an important step in discovering the new anticancer agent that has no adverse side effects. The test showed that PB aqueous extract caused no significant toxicity on HGF-1 even at the highest concentration tested, thus it can be considered safe to be used on normal cell. Therefore, an agent that can selectively induce cell death in cancer cells with low cytotoxicity to the normal cells would be a potential anticancer agent against cancer (Shang et al., 2012).

CONCLUSION

PB aqueous extract contains phenolic compounds and works in a dose dependent manner towards A375 cell line (Skin Malignant Melanoma) tested. In anti-proliferative assay, it is evident that PB extract inhibit the growth of cancer cells when compared to untreated. PB extract demonstrated that it exert cytotoxic and

cytostatic on A375 but not toward normal cells even at highest concentration tested. Therefore, PB aqueous extract might be considered a potential anticancer agent, however further research studies are still required to explore its insight mechanism of action as an anticancer agent.

ACKNOWLEDGEMENT

This research work was financially supported by through shared Research Grant Scheme of FRGS 13-055-0296, FRGS 16-045-0554, FRGS 16-046-0545 and RIGS 15-085-0085. Special thanks to Jabatan Perlindungan Hidupan Liar & Taman Negara (PERHILITAN) Semenanjung Malaysia (Department of Wildlife and National Parks Peninsular Malaysia) for supporting and issuing the research permit. Also thanks to research laboratory staff of Integrated Centre for Research Animal Care and Use (ICRACU) as well as to Kuliyyah of Medicine, Kulliyyah of Pharmacy and Kuliyyah of Allied Health Science, IIUM, Kuantan, Malaysia for providing all research facilities to accomplish this research work.

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