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ORIGINAL ARTICLE

Tannic Acid-Rich Porcupine Bezoars Induce Apoptosis and Cell Cycle Arrest in Human Colon Cancer Cells

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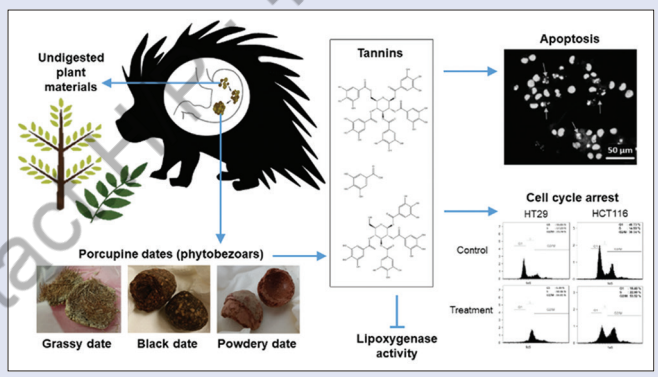
ABSTRACT

Background: Porcupine bezoar, a phytobezoar used as traditional medicine, was recently claimed to effectively treat cancer. However, there is a lack of scientific evidence to prove the claim. **Objectives:** This study aimed to scientifically examine porcupine bezoars as a potential anticancer agent and to investigate their principal bioactive constituents. **Materials and Methods:** The porcupine bezoars were extracted using methanol and further Sephadex LH-20 column chromatography was used to enrich the tannins content. The inhibitory effects of the crude extracts on a panel of cancer cell lines were first determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Then, the anticancer activities of the enriched fractions in selected cell lines were analyzed, while the chemical composition of the active fraction was identified using liquid chromatography—electrospray ionization-tandem mass spectrometry. **Results:** Crude extracts of black date and powdery date effectively inhibited colon cancer cell lines HT-29 and HT-116, but not the normal colon cells, and their tannin-enriched fractions demonstrated higher inhibitory effects when compared to the extracts. Further, the fractions arrested cell cycle at S phase and induced apoptosis in treated colon cancer cells with a similar effect to that of commercial tannic acid. Lipoxygenase activity which plays a role in tumorigenesis of colon cancer was also inhibited by these fractions. Chemical analysis found that both the enriched fractions and commercial tannic acid share similar chemical constituents, including gallic acid and its derivatives (polygalloyl glucose). **Conclusion:** Together, the results suggest that tannic acid in porcupine bezoars may inhibit colon cancer cells by interfering cell proliferation and triggering program cell death in the cells.

Key words: Apoptosis, cell cycle arrest, colon cancer, porcupine date, tannic acid

SUMMARY

- Animal phytobezoars were extensively used in the early modern era, and some were recorded in the ancient pharmacopeia. Porcupine bezoars, previously named as *Lapis malaccensis* by Caspar Bauhin, are phytobezoars found in the gall bladder/stomach of Himalayan porcupine (*Hystrix brachyura*). Lately, porcupine bezoars gained popularity among Asian Chinese in the 20th century. Many testimonies stated that it can cure various illnesses including cancer, but there is a lack of scientific proof for the claim. In collaboration with a licensed herbal supplier in Malaysia, porcupine bezoars were obtained legally for testing of their pharmacological activities. This article summarized our findings on the effectiveness of porcupine bezoar in treating various cancer cell lines and the major bioactive constituent that contributes to its cytotoxic activities. One unique feature of our research is the enrichment of tannins in bezoar extract led to significantly enhanced toxicity in tested colon cancer cells, and further analysis shows the enriched fraction shares highly similar chemical composition and cytotoxic activities with commercial tannic acid, a specific form of tannin. The findings described in this preliminary study provide important evidence for further investigation on the pharmacological action of porcupine bezoar.



Abbreviations used: BD: Black date; BDTF: Black date tannins fraction; DMSO: Dimethyl sulfoxide; GD: Grassy date; HPLC: High-performance liquid chromatography; MS: Mass spectrometry; PD: Powdery date; PDTF: Powdery date tannins fraction; PGG: 1, 2, 3, 4, 6 penta-O-galloyl-β-D-glucose; Q-TOF: Quadruple time of flight; RT: Retention time; SI: Selective index; TCM: Traditional Chinese Medicine.

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INTRODUCTION

Porcupine bezoars, another type of medicinal bezoar stones, are composite of plant-derived phytobezoars formed in the gastrointestinal lining of Himalayan porcupine, *Hystrix brachyura*. These bezoars are well known among Asian Chinese for their claimed effectiveness in treating inflammation, dengue fever, and acute pain, and many believed that these bezoars could treat cancers. The types of porcupine bezoars and their properties were recently reviewed.[1] However, most of the pharmaceutical properties of porcupine bezoars were registered based on patient testimonies.[2] There is a lack of scientific evidence to support the claim; and hence, this represents a critical area to be investigated so that a comprehensive medicinal knowledge on porcupine bezoars can be established to prevent misprescription of bezoar-based treatments, especially for cancer patients. Several types of porcupine bezoar

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dates were previously investigated and tannins were discovered as the main constituent (≈ 93% of the total phenolic content).[3] In recent years, increasing evidence suggest that tannins isolated from plants possess anticarcinogenic and antiproliferative effects both *in vitro* and *in vivo*.[4-6] Therefore, it is hypothesized that tannins as major component in the porcupine bezoars may exert significant effects in inhibiting proliferation and inducing apoptotic cell death in cancer cells. The aim of this study is to investigate the cytotoxic effects of three porcupine bezoars: grassy date (GD), black date (BD), and powdery date (PD) in various cancer cell lines. Further, tannin-enriched fractions of the bezoars which showed significant cytotoxicity were examined for their effects on induction of apoptosis and inhibition of cell cycle progression in colon cancer cells.

MATERIALS AND METHODS

Preparation of crude extracts and fractions

Finely ground raw sample of porcupine bezoar was added into 100% methanol (1:50, w/v) and shaking for 1 h using orbital shaker (200 rpm) at room temperature. The methanol extract was collected by filtration, and the residues left on the filter were extracted two more times with 100% methanol. The three resulting crude extracts were then pooled and lyophilized. Sephadex LH-20 column chromatography was used to prepare tannin-enriched fraction from the crude extract. First, methanol crude extracts were reextracted into ethanol soluble fraction using absolute ethanol to remove undesired compounds such as protein and nonphenolic phytochemicals. Two mobile phases (phase A: 95% EtOH: 5% H_2O; and phase B: 70% aceton: 30% H_2O) were utilized to separate the ethanol fraction into BD tannins fraction/PD tannins fraction (BDTF/PDTF) and BD nontannins fraction/PD nontannins fraction (BDNTF/PDNTF). The three-column volume of Mobile phase A was first run through the column and the eluted samples were collected as NTF. Then, the three-column volume of Mobile phase B was used to elute tannins bound to the Sephadex LH-20 column. Eluted samples were then lyophilized.

Cell lines and culture methods

Cell lines H400 (oral carcinoma), MCF-7 (breast carcinoma), HT-29 (colon adenocarcinoma), HepG2 (liver carcinoma), SHSY5Y (neuroblastoma), HCT-116 (colon adenocarcinoma), SW480 (colon adenocarcinoma), and CCD-841-Con (normal colon) were purchased from American Type Culture Collection (ATCC, Manassas, USA). Media and supplements for culturing each of these cell lines are listed in Table A1 in Appendices. All the cell lines were maintained at 37°C with 5% carbon dioxide.

Cell viability assay

Cells were cultured in 96-well plates at density of 5000–12,000 cells per well and allowed to attach overnight, then treated with vehicle (dimethyl sulfoxide) and indicated concentration of porcupine date extracts (BD, PD, BDTF, or PDTF) for 48 h. After treatment, cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay adopted from the previous study.[7] Briefly, cells were incubated with 5 mg/ml of MTT in PBS for 2–4 h and then removed and replaced with 100% DMSO to solubilize the formazan crystal. Absorbance was measured at 570 nm using Tecan Infinite 200 microplate reader (Tecan Life Sciences, Switzerland). All cell viability assays were performed in triplicate.

Cell proliferation assay

Cells were cultured in 96-well plates at density of 5000 cells per well and allowed to attach overnight, then treated with indicated concentration of BD and PD for 48 h. Proliferation index was then quantified using BrdU cell proliferation enzyme-linked immunosorbent assay (Roche, Swiss) according to manufacturer protocol. Briefly, 10 µl of BrdU labeling solution was added into each well and incubated for 24 h, then fixed with 50 µl of acidified ethanol for 30 min at room temperature. About 100 µl of anti-BrdU-POD solution was then added into each well to stain the cells for 90 min. Stained cells were washed with PBS and then developed with 100 µl substrate solution for approximately 30 min. 25 µl 1M H_2SO_4 was added to stop the reaction. Absorbance at wavelength 450 nm (reference wavelength: 690 nm) was measured using Tecan Infinite M200 microplate reader (Tecan Life Sciences, Switzerland). Proliferation index was calculated based on the percentage of treated cells compared to untreated control.

Cell cycle analysis

Approximately 5×10^5 cells were seeded into a 6-well plate and allowed to attach overnight, then treated with the indicated concentration of sample fractions (BDTF and PDTF) and tannic acid for 24 h. Treated cells were trypsinized and washed with PBS. Cells were then fixed with ice cold 70% ethanol for 30 min at 4°C. Fixed cells were washed and resuspended in 100 µl staining buffer containing 5 µl of RNase and 15 µl of 7-Aminoactinomycin D (7-AAD) solution. Fluorescence emitted from 7-AAD-stained nucleus was detected using Amnis ImageStream flow cytometry with 488 nm filter within 1 h. Data were analyzed using IDEAS 6.2 software (Merck, USA).

Imaging analysis

Cells were first fixed with 4% paraformaldehyde for 30 min and subsequently stained with Hoechst 33258 (5 µg/mL) for 1 h at room temperature. Stained cells were resuspended in 100 µl of PBS: glycerol (1:1) and photographed using UV fluorescent microscope to identify apoptotic nuclei morphology.

Apoptosis assay

Cells were stained with Annexin V and propidium iodide (PI) according to the manufacturer protocol (FITC Annexin V/PI apoptosis detection kit, BD Biosciences). Briefly, approximately 5×10^5 cells per well were seeded into a 6-well plate and allowed to attach overnight, then treated with the indicated concentration of sample fractions (BDTF and PDTF) and tannic acid for 24 h. Treated cells were washed twice with ice-cold PBS and resuspended in 100 µl binding buffer (0.1 M Hepes/NaOH pH 7.4, 1.4 M NaCl, and 25 mM $CaCl_2$). Then, 5 µl of Annexin V-FITC and 5 µl of 100 µg/ml PI solutions were added to the cells and incubated in the dark for 15 min. After incubation, 400 µl of binding buffer was added to the stained cells before analyzed using Amnis ImageStream flow cytometry with 488 nm filter within 1 h. Data were analyzed using INSPIRE and IDEAS 6.2 software (Merck, USA).

Measurement of lipoxygenase inhibitory activity

Inhibition of lipoxygenase (LOX) activity was determined according to the method as described by Azhar-Ul-Haq *et al.*[8] with slight modification. Briefly, extracts (BD, PD, BDTF, PDTF, BDNTF, and PDNTF) were dissolved in DMSO and diluted to 100 ug/mL with 100 mM phosphate buffer (pH 8.0) together with soybean LOX Type-1B solution. Enzyme reaction was initiated by addition of linoleic acid (substrate) solution and incubated at 25°C for 10 min. The conversion of linoleic acid to form 13(S)-Hydroperoxyoctadeca-9Z,11E-dienoic acid (13-HPODE) was measured at 234 nm. Nordihydroguaiaretic acid (NDGA) was used as positive control. Dose–response curve was plotted to determine the inhibition concentration at 50% (IC_{50}) using Microsoft Excel.

Identification of compound in tannins fraction

The chemical profiles of ethanol fraction and the resulting BDTF/PDTF and BDNTF/PDNTF fractions along with gallic acid (Fluka >98%, USA) and tannic acid (Sigma T-8406, USA) standard were evaluated using high-performance liquid chromatography (HPLC). Fractions were dissolved in 10% MeOH, and chromatographic separation was done using Agilent Poroshell 120 EC-C_{18} (2.1 mm × 100 mm, 2.7 μm) column. The mobile phase used was 100% water (solvent A) and 100% methanol (solvent B). The optimized runtime was: 10%–27% B from 0 to 25 min, 27%–45% B from 25 to 70 min, 45%–100% B from 70 to 80 min, and 100% B from 80 to 90 min. Liquid chromatography-mass spectrometry (LC-MS) was then performed using Agilent 1290 Infinity LC system with addition of 1% formic acid to the solvent systems. The compounds were putatively assigned based on their retention times (RTs) and m/z values from the LC-MS results. TF (BDTF/PDTF) and tannic acid were further identified using LC-electrospray ionization (LC/ESI)-MS/MS using preoptimized chromatography conditions. ESI-MS/MS(−) were obtained using Agilent 6520 Accurate-Mass Quadruple time of flight mass spectrometer with dual-ESI source. The MS fragments of the compounds were matched with online databases (Metlin, MassBank, SciFinder, HMDB, and FoodDB).

Statistical analysis

The data were subjected to statistical analysis using two-way ANOVA followed by Tukey's multiple comparison test; unless otherwise stated. All analyses were performed using Prism Windows 7.02 (GraphPad Software, San Diego, USA). Results are expressed as the mean ± standard error mean of three independent experiments in triplicate with $P < 0.05$ as statistically significant.

RESULTS

Effect of porcupine date crude extracts on viability of various cancer cells

MTT assay was performed to determine the cytotoxic effects of methanol crude extracts of GD, BD, and PD in various cell lines. The concentrations of each extract which inhibited 50% of cancer cells (H400, MCF-7, HepG2, SHSY5Y, HCT-116, HT-29, and SW480) and normal cell line (CCD-841-CoN) in comparison to control (IC_{50}) at 48 h are depicted in Table 1. Within the tested concentration (0–200 μg/ml), H400 (oral), MCF-7 (breast), and HepG2 (liver) cancer lines maintained their decent viability (>50% compared to control) when treated with all three type of date extracts, while GD induced insignificant toxicity in all tested cell lines. BD extract was found effective against brain cancer cell SHSY5Y (IC_{50} = 80 μg/ml) and two lines of colon cancer (HCT-116, IC_{50} = 75 μg/ml; HT-29, IC_{50} = 70 μg/ml), while PD extract exerted inhibitory effect on viability of all three tested colon cancer cell lines, with a range of IC_{50} 95-190 μg/ml. It is worth noting that extracts of both BD and PD induced negligible toxicity in normal colon cell CCD-841-CoN within tested concentration (200 μg/ml) [Table 1].

Table 1: Inhibitory effects of methanol extracts of porcupine bezoars (48 h)

Cell lines	IC_{50} (μg/mL)		
	GD	BD	PD
H400 (oral)	>200	200	>200
MCF-7 (breast)	>200	>200	>200
HepG2 (liver)	>200	>200	>200
SHSY5Y (brain)	>200	80	>200
HCT-116 (colon)	>200	75	100
HT-29 (colon)	>200	70	95
SW480 (colon)	>200	>200	190
Normal cell lines			
CCD-841-CoN (colon)	>200	>200	>200

GD: Grassy date; BD: Black date; PD: Powdery date; IC_{50}: Concentration of each extract which inhibited 50% of cells in comparison to control

Antiproliferative and apoptotic effects of porcupine date crude extracts

Extracts of both BD and PD showed selective toxicities on colon cancer cells. Therefore, their inhibitory effects on the growth of HT-29 and HCT-116 cell lines were further investigated using BrdU assay and apoptotic nuclear stain. DNA synthesis, which can be measured by BrdU labeling, is an indicator of cell proliferation rate.[9] Both extracts at the concentration of 100 μg/ml significantly reduced 30%–40% of proliferation index of two tested colon cancer cell lines. At a higher dose (200 μg/ml), HT-29 showed a sensitive response in porcupine date treatment with a proliferation index below 20% [Figure 1a], while reduced proliferation of HCT-116 remains ~50% [Figure 1b]. During apoptotic execution, chromatin undergoes a phase change from a heterogeneous, genetically active network to an inert highly condensed form that is fragmented and packaged into apoptotic bodies.[10] When the nucleus of HT-29 cells was stained, condensed chromatin with some was fragmented as apoptotic nuclei morphology were observed in those cells treated with the extracts of BD and PD at their concentrations of IC_{50}, respectively, for 24 h [Figure 1c].

Enhanced cytotoxicity of tannin-rich fraction of porcupine date extracts

Our previous study showed 93% of the total phenolic content in BD and PD is hydrolyzable tannins, the polyphenols which tend to hydrolyze into glucose and gallic or ellagic acid units.[11] In this study, TF and NTF obtained using Sephadex LH-20 column chromatography were first analyzed using HPLC. HPLC profiles of TF and NTFs of both BD (BDTF and BDNTF) and PD (PDTF and PDNTF) together with standard compounds gallic acid and tannic acid were shown in Figure 2a. The peak at RT 6.5 min (peak no. 1) that shows the presence of gallic acid was detected in TF (BDTF/PDTF) and commercial tannic acid, while its presence in NTF (BDNTF/PDNTF) is insignificant. Multiple peaks at higher RT (peaks no. 2–16) were observed in the chromatograms of standard tannic acid and TFs of both BD and PD [Figure 2a]. LC-MS analysis indicated that these peaks might imply the presence of a mixture of polygalloyl glucose containing two to five gallic acid residues per molecule [Figure 2b]. These profiles were not observed in NTF [Figure 2]. Together these results confirm the enrichment of hydrolyzable tannins in TF. Next, toxicities of these fractions in colon HT-29 and HCT-116 cancer cell lines were tested. Apparently, enrichment of tannins in porcupine date extracts (BDTF and PDTF) significantly enhanced their inhibitory effects on viability of both tested cell lines from doses as low as 25 μg/ml to the highest tested concentration 200 μg/ml [Figure 3a and b]. The values of IC_{50} reflecting toxicities of the crude extracts as well as the fractions were summarized [Figure 3c]. In general, tannins-enriched fractions of both BD and PD extracts showed remarkable higher toxicities in all tested cell lines with IC_{50} 45–55 μg/ml when compared to that of crude extracts (IC_{50} 70–100 μg/ml) and NTFs (IC_{50} 110–>200 μg/ml).

Apoptotic effect of tannin-rich fraction of porcupine extracts

Apoptosis induction by BDTF and PDTF was analyzed using flow cytometry with Annexin V/PI staining. Cells treated with commercial tannic acid and camptothecin were analyzed at the same time as the reference and the positive control, respectively. Early apoptosis is

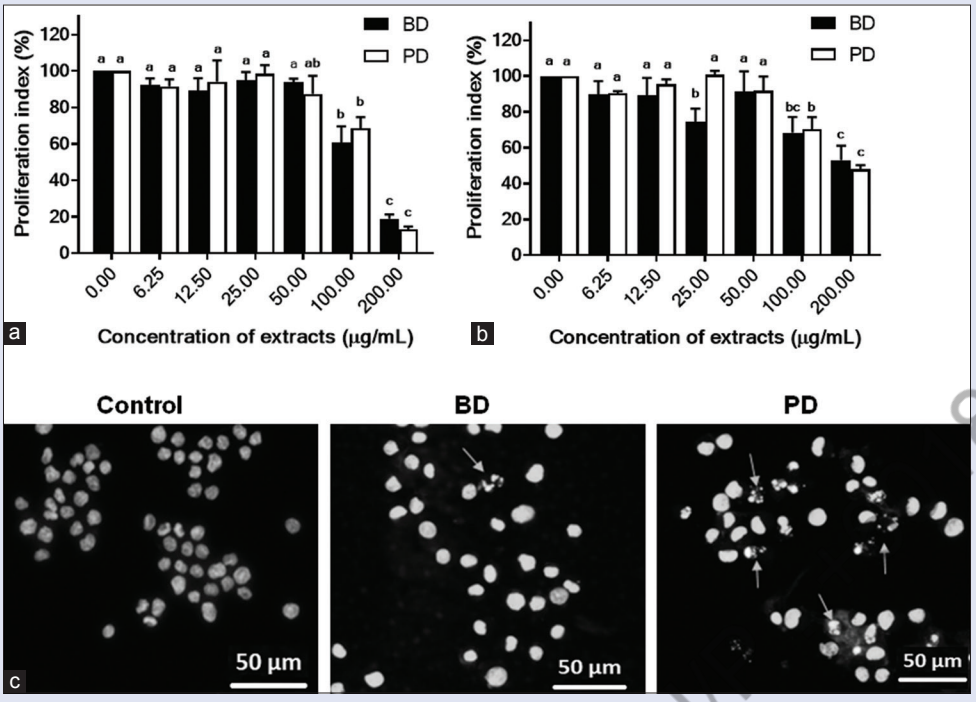


Figure 1: Antiproliferative effects of black date and powdery date on (a) HT-29 and (b) HCT-116 cells. Cells were treated with black date and powdery date at concentration range between 6.25 and 200 μg/ml for 48 h. BrdU Proliferation Index was calculated based on percentage compared to untreated control. Results were expressed as mean ± standard error mean of three independent experiments (n = 3). Bars with different letters represent significant differences (P < 0.05). (c) Hoechst-stained nucleus of HT-29 in untreated control, black date (70 μg/ml), and powdery date (100 μg/ml). Arrows indicate the apoptotic nucleus

characterized by the occurrence of externalization of phosphatidylserine due to cell membrane flipping, while membrane remains impermeable to PI (Annexin V+, PI–).[12] In this study, we observed significantly higher percentage of early apoptotic cells in BDTF- and PDTF-treated HCT-116 cells (24% and 35.67%, respectively) [Figure 4a and c]. In late apoptotic cells, the integrity of the plasma and nuclear membranes compromises, allowing PI to pass through the membranes, intercalate into nucleic acids and display red fluorescence (Annexin V+, PI+).[12] Up to 26.10%–31.76%-treated HT-29 cells were detected with significant level of late apoptosis when compared to control group (3.03%) [Figure 4a and b]. However, 19.92% of untreated HCT-116 control cells were observed with late apoptosis induction. Among all the treatment groups, only those treated with 100 μg/ml PDTF (26.07%) and 100 nM camptothecin (27.45%) showed significant higher level of late apoptosis induction when compared to control group [Figure 4c]. In general, flow cytometric dot distribution of both HT-29 and HCT-116 cells treated with tannins-enriched fractions of both BD and PD resembles the dot plots of those treated with commercial tannic acid, implicating these treatments may induce similar type of program cell death.

Inhibitory effect of tannin-rich fraction of porcupine date extracts on cell cycle progression

Regulation of cell cycle through the G1/S and G2/M checkpoints is a crucial event for cell proliferation, and the checkpoints are potential targets for many anticancer drugs. To further investigate the antiproliferative effects of BD and PD on colon cancer cells, DNA content in treated HT-29 and HCT-116 cells was measured using 7-AAD staining and flow cytometry. In HT-29 cells without any treatment, 55.65% contained 2N amount of DNA (G1 phase) and 23.74% with 4N of DNA (G2/M phase), while only 17.23% of cells were synthesizing their DNA (S phase). Intriguingly, exposure to 100 μg/ml of BDTF and PDTF strikingly increased proportion of HT-29 cells with S phase (50.36% and 55.90%, respectively), which was ensued with equivalent decrease in proportion of cells with G1 phase (5.34% and 6.58% respectively). However, there was no apparent impact of the same dose of tannic acid on cell cycle progression of HT-29 cells [Figure 5a and b]. Distribution of control HCT-116 cells at different phases of cell cycle (G1: 46.73%, S: 14.90%, G2/M: 36.34%) was similar to those observed in untreated HT-29 cells, while the treatments of BDTF and PDTF as well as tannic acid significantly increased HCT-116 cells with G2/M phase (53.52%–58.38%) accompanied by a decrease of cells with G1 phase (12.47%–22.45%), implicating tannins-enriched fractions and tannic acid standard induce G2/M arrest in HCT-116 cells [Figure 5a and c]. Nonetheless, the most apparent G2/M arrest was observed in the treatment of camptothecin (100 nM) for both HT-29 and HCT-116 cells [Figure 5].

Inhibitory effect of porcupine date extracts on lipoxygenase activity

LOXs are a versatile class of oxidative enzymes involved in arachidonic acid metabolism linking cancer and inflammation.[13] In this study, an *in vitro* assay was employed to examine the inhibitory effect of porcupine date extracts on LOX activity that metabolizes linoleic acid to form 13(S)-Hydroperoxyoctadeca-9Z,11E-dienoic acid (13-HPODE). Methanol crude extracts of both BD and PD showed some enzyme inhibition, which was 24.78% ± 1.86% and 29.48% ± 1.95%, respectively, nearly two times higher than the inhibitory effects observed in NTFs (BDNTF/PDNTF) (9.39% ± 2.29% and 15.50% ± 1.93%). Significant higher inhibitory activities were observed in tannin-rich fractions (BDTF and PDTF), 73.04% ± 2.98% and 88.73% ± 5.75%, respectively, which are

comparable to those of commercial tannic acid (96.02% ± 1.69%) and positive control NDGA (97.94% ± 1.12%) [Table 2].

Identification of compound in tannins fraction of porcupine bezoar

The fragmentation pattern of BDTF and PDTF was investigated using ESI tandem MS (ESI-MS/MS). Tannic acid (Sigma T-8406) was included as a reference. The ESI-MS/MS analysis of tannins-enriched fractions and tannic acid allowed us to identify a series of gallic acid and tannin-related polygalloyl glucoses [Tables A2-A4]. The identified compounds are summarized in Table 3. Both LC-MS and ESI-MS/MS analyses confirmed that tannins-enriched fractions of both BD and PD as well as standard tannic acid contain a mixture of several hydrolyzed forms of tannins with varying number of galloyl groups [Figure 2b and Table 3]. It is noted that in LC-MS analysis, for a measured m/z value, several galloyl glucose derivatives could be assigned, dependent on how the galloyl groups are positioned in the structure [Figure 2]. ESI-MS/MS mass fragments matching identified gallic acid, tetrakisgalloyl, and pentakisgalloyl glucoses were identified in all the samples, whereas digalloyl and trigalloyl glucoses were observed only in PDTF and tannic acid [Table 3].

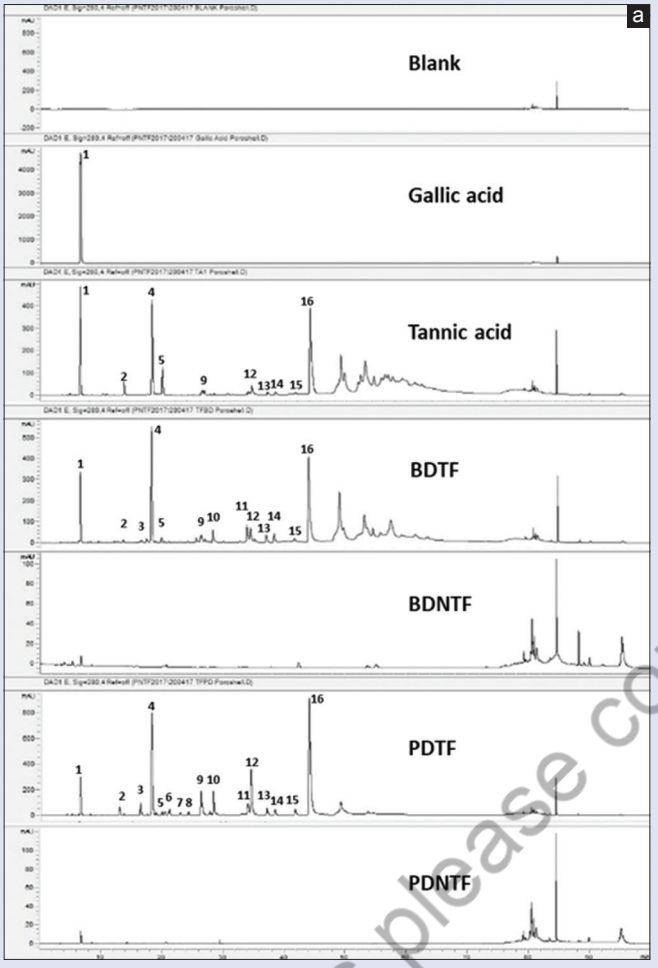


Figure 2: (a) High-performance liquid chromatography profiles of tannins fraction and nontannins fraction of black date and powdery date separated using Sephadex LH-20 column chromatography. (b) List of putative compounds identified using liquid chromatography-mass spectrometry analysis

Compound (s) [Figure 2a]	Putative identity	HPLC-DAD λ_{max} (nm)	HPLC-ESI (-)-MS (m/z)
1	Gallic acid	280	169.01422
2	Digallate	280	321.0261
3	Pedunculagin	280	783.06996
4, 5, 6, 7, 8	1,3,6-Trigalloyl-beta-D-glucose	280	635.09024
9, 10, 12, 15	1,2,3,6-Tetrakis-O-galloyl-beta-D-glucose	280	787.1006
11, 13, 14, 16	1,2,3,4,6-Pentakis-O-galloyl-beta-D-glucose	280	469.05361 $(M-2H)^{-2}$

HPLC: High-performance liquid chromatography; ESI: Electrospray ionization; MS: Mass spectrometry

DISCUSSION

In our previous study, crude extract of porcupine bezoar showed significant *in vitro* and intracellular antioxidant activity. In addition, hydrolyzable tannin which was identified as the major compounds in porcupine bezoars was reported to possess anticancer activity.[3] To further elucidate the claim on the anticancer properties of these bezoars, their cytotoxic activities on a series of cancer cell lines were first screened in this study. The crude extracts from BD and PD were found, particularly effective in inhibiting viability of colon cancer cell lines HT-29 and HCT-116, with insignificant cytotoxicity observed in nontumorigenic colon CCD-841-CoN cells. When Selective Index (SI) was calculated by comparing IC_{50} values of porcupine extracts in non-tumorigenic cells against the IC_{50} of the same extracts in cancer cell lines, selectivity of these extracts against colon cancer cells was significant with SI value >11.43, exceeding the agreed criteria for the (SI >10) used in a drug discovery program.[14] All three colon epithelial cancer cell lines (HT29, HCT116, and SW480) tested in this study are different in appearance and growth characteristic. Both HT-29 and HCT-116 are fast-growing cells which were found sensitive to the treatment of porcupine bezoar extracts, while SW-480 cells with a slower growth rate showed insignificant response in the treatment. The varied responses could be due to the differentially regulated metabolic pathway in these cancer cells and/or the distinct genetic and epigenetic background of different cell lines.[15,16] In the study on porcupine bezoars, GD was initially investigated in parallel with BD and PD. However, GD showed relatively low antioxidant activity in our previous study[3] and limited cytotoxicity of GD against all tested cancer cell types was also observed in this study. Hence, BD and PD which showed selective inhibitory effects on colon cancer cells were focused for more in-depth investigation.

In this study, the cytotoxic activities of hydrolyzable tannins in BD and PD were verified by comparing the effects of extract fractions with or

Table 2: Inhibitory effect of different extracts/fractions of porcupine dates on lipoxygenase activity

Samples[a]	Lipoxygenase inhibition (%)[b]
Methanol extract of black date	24.78±1.86
Methanol extract of powdery date	29.48±1.95
BDTF	73.04±2.98
PDTF	88.73±5.75
BDNTF	9.39±2.29
PDNTF	15.50±1.93
Tannic acid standard	96.02±1.69
Nordihydroguaiaretic acid (NDGA)	97.94±1.12

[a]All the samples were tested at the concentration of 100 μg/ml; [b]All values are mean±SEM of three independent experiment. 100% mean fully inhibited activity. Nordihydroguaiaretic acid was used as positive control in this assay. BDTF: Black date's tannins fraction; PDTF: Powdery date's tannins fraction; BDNTF: Black date's nontannins fraction; PDNTF: Powdery date's non-tannins fraction

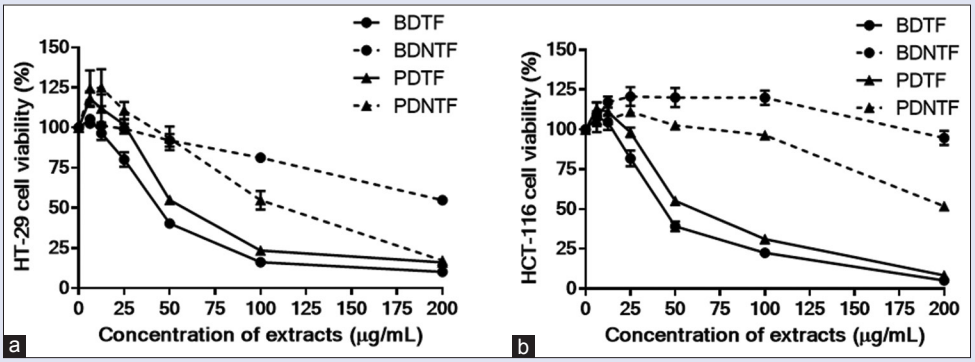


Figure 3: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to evaluate dose-dependent cytotoxicity effects of tannins fraction and non-tannins fraction of black date and powdery date on (a) HT-29 and (b) HCT-116 cells for 48 h. (c) Summary of half maximal inhibitory concentration at 50%

Fractions	IC_{50} (μg/ml)	
	HT-29	HCT-116
BD methanol crude extract	75	70
BDTF	46	45
BDNTF	>200	>200
PD methanol crude extract	100	95
PDTF	55	55
PDNTF	110	>200

BD: Black date; PD: Powdery date; BDNTF: Black date's non-tannins fraction; PDTF: Powdery date's non-tannins fraction; BDTF: Black date's tannins fraction; PDNTF: Powdery date's nontannins fraction; IC_{50}: Concentration of each fraction which inhibited 50% of cells in comparison to control

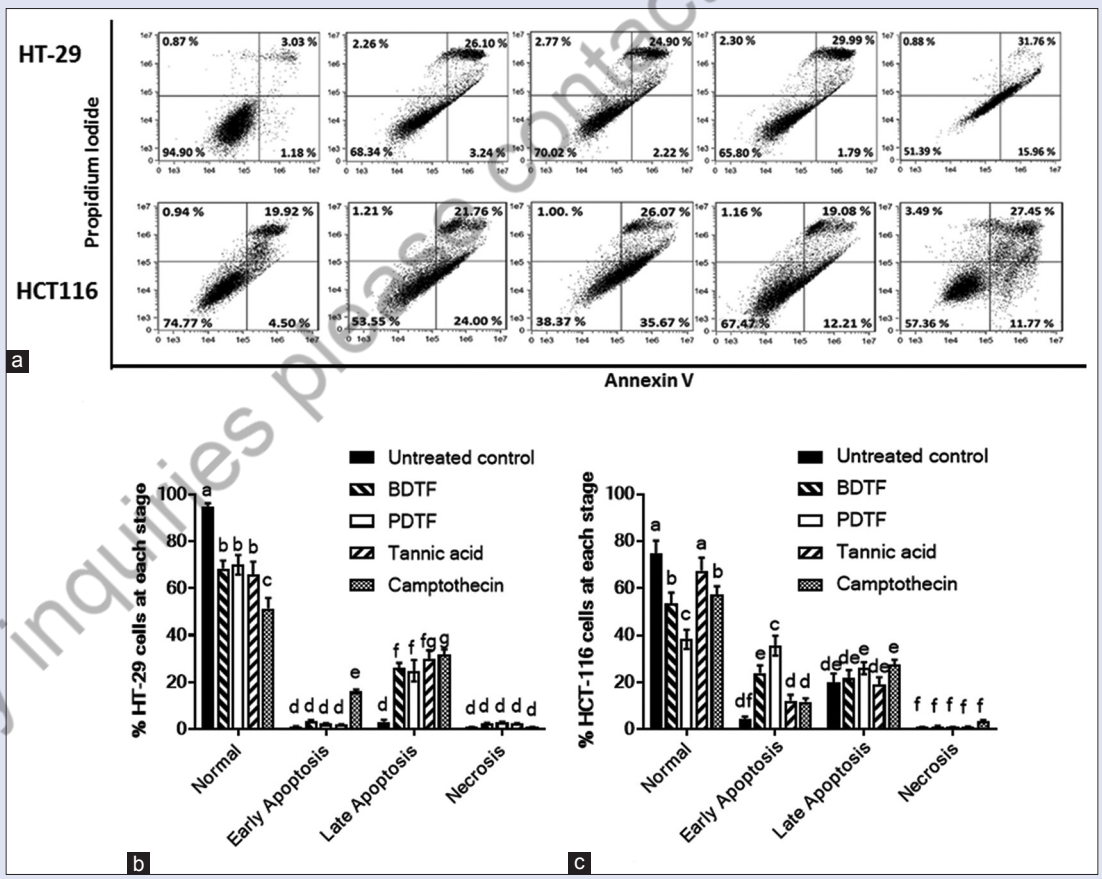


Figure 4: (a) Flow cytometry analysis to evaluate apoptosis induction in HT-29 and HCT-116 cells treated with 100 μg/ml of tannins fraction of black date and powdery date and tannic acid or 100 nM camptothecin control for 24 h. Percentage of apoptotic (b) HT-29 and (c) HCT-116 cells in various treatments were compared. Results were expressed as mean ± standard error mean of three independent experiments ($n = 3$). Bars with different letters represent significant differences ($P < 0.05$)

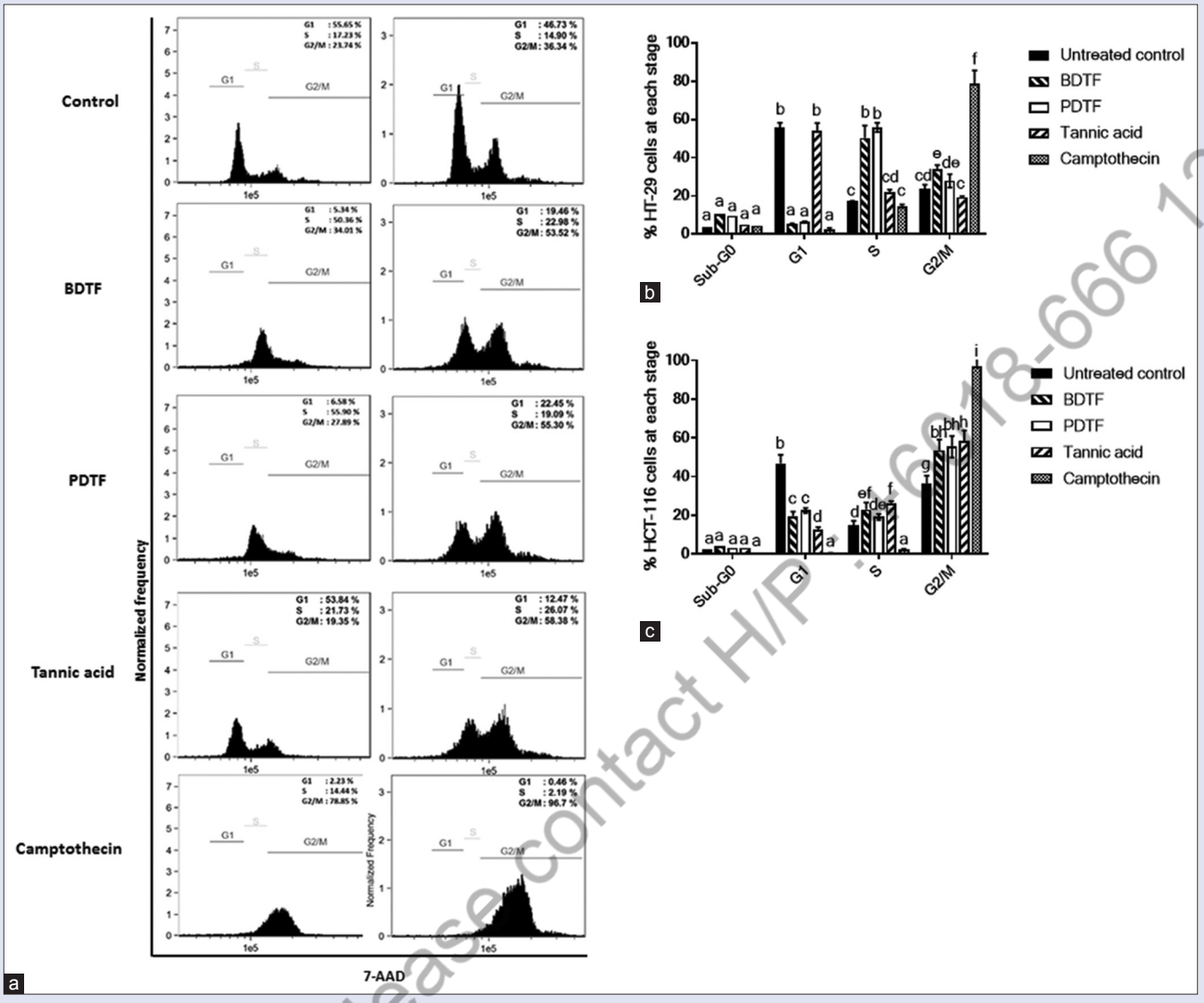
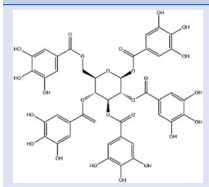
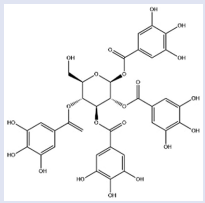
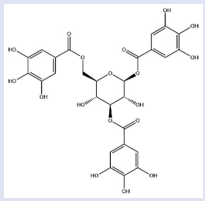
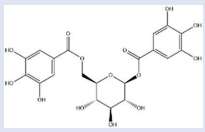
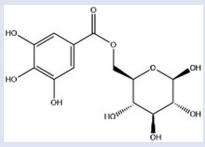
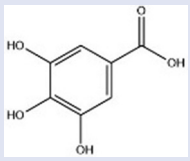


Figure 5: (a) Flow cytometry analysis of 7-AAD-stained cellular DNA to evaluate cell cycle progression in HT-29 and HCT-116 cells treated with 100 μg/ml of black date tannins fraction, powdery date tannins fraction and tannic acid or 100 nM camptothecin control for 24 h. Distribution of (b) HT-29 and (c) HCT-116 cells in different phases of cell cycle were compared. Results were expressed as mean ± standard error mean of three independent experiments ($n = 3$). Bars with different letters represent significant differences ($P < 0.05$)

without enrichment of tannins. The most common type of hydrolyzable tannin, tannic acid was included as a reference in the study. Tannic acid is referred as a hydrolyzable decagalloyl glucose macromolecule (MW: 1701.19 g/ml) with a core glucose structure esterified with ten galloyl groups. In tannic acids, the depside bonds between galloyl groups are less stable than the aliphatic ester bonds between galloyl groups and the core glucose. The hydrolyzable property of tannic acid tends to cause the compound to generate methyl gallate, gallic acid, and several polygalloyl glucoses in the experiment, depending on the number of galloyl moiety being hydrolyzed.[17] There is no surprise that commercial tannic acid is actually a mixture of dozens of closely related yet different chemicals. HPLC profiles and LC-MS analysis validated the chemical constituents (gallic acid and polygalloyl glucoses) which resemble those in commercial tannic acid were enriched in tannin-enriched fractions of both BD and PD (BDTF and PDTF). It appears that these tannin-enriched fractions showed 1.5–2.0 folds higher cytotoxic activities in tested colon cancer cells, while minimal effect was observed for NTFs suggesting that tannins are the major bioactive compounds in the porcupine dates BD and PD. Nevertheless, some cytotoxic effects of NTF of PD on HT-29 cells (IC_{50} 110 μg/ml) were noted in this study. These effects could be the attributes of nontannin compounds such as flavonoids, terpenoids, and cardiac glycoside which were identified as the chemical constituents of porcupine bezoars (besides tannins) in our previous study.[3]

The accurate transition of cells across different phases of the cell cycle is crucial for the proliferation of eukaryotic cells.[18] However, the regulation of cell cycle in cancer is compromised due to genetic mutation resulting in uncontrolled cell proliferation. Therefore, checkpoints that control cell cycle from improper progression are targets for many anticancer agents.[19-21] Crude extracts of both black and PDs showed significant antiproliferative effects in tested colon cancer cell lines. Further, explicit high percentage of cells with S phase in HT-29 cells treated with tannins-enriched fractions of BD and PD were observed, while these fractions and tannic acid triggered G2/M arrest in HCT-116 cells. The

Table 3: Identification of gallic acid and its derivatives in black date, powdery date, and tannic acid using electrospray ionization-mass spectrometry/mass spectrometry

Structures	Gallic acid and its derivatives	Number of galloyl	Samples*		
			BD	PD	Tannic acid
	1,2,3,4,6-Penta-O-galloyl-β-D-glucose	5	+	+	+
	1,2,3,6-Tetrakis-O-galloyl-β-D-glucose	4	+	+	+
	1.3.6-Trigalloyl- β-D-glucose	3	-	+	+
	Digalloyl glucose	2	-	+	+
	Glucogallin	1	-	-	-
	Gallic acid	-	+	+	+

*The compound (gallic acid or its derivatives) was identified (+) or not identified (-) in the samples (extracts of BD/PD or tannic acid standard) analyzed using LC-ESI-MS/MS. BD: Black date; PD: Powdery date; ESI: Electrospray ionization; MS: Mass spectrometry

intra-S-phase checkpoint response to DNA damage slows down the cell-cycle progression to activate DNA repair system.[22] Tannins from porcupine bezoars may cause DNA damage in HT-29 cells, but interfere microtubule stability in HCT-116 cells as tannic acid was reported to induce G2/M phase arrest in Hep3B cells with interesting effect on the cytoskeleton.[23] Unrecoverable cell cycle progression or severe toxicity caused by therapeutic agents could induce apoptosis in treated cancer cells. Hydrolyzable tannins such as ellagitannins were reported to induce apoptosis in HT-29 cell lines.[24] At the same concentration of 100 μg/ml, the apoptosis-inducing effect of tannins-enriched fractions of BD and PD was found to be identical to that of tannic acid in the colon cancer cells tested in this study.

LOX and their arachidonic acid-derived eicosanoid products are known to regulate cell growth, survival, migration, and invasion in cancers,[13] and 5-LOX is specifically highly upregulated in colon cancers.[25] NDGA used in our study was well studied as LOX inhibitor, and this compound was also shown to suppress the growth of various cancer cells.[26-28] Interestingly, tannins in BD and PD showed remarkable inhibitory effects on *in vitro* LOX activity which are comparable to the effect of NDGA. In line with the above described cell-cycle arrest and apoptosis induction, cytotoxic activities of porcupine bezoars-related tannins in colon cancer cells may also involve LOX inhibition and possibly attributed to the gallic acid derivatives (polygalloyl glucoses) that were identified in the enriched fractions of BD and PD. A recent study by Tanimura *et al.*[29] suggested that the activity of hydrolyzable tannins is related to the side group attached to the central glucose. Results from both measured m/z value and mass fragment matching support that the tested porcupine bezoars contain gallic acid and its derivatives especially those with high number of galloyl groups, e.g., 1,2,3,4,6-pen ta-O-galloyl-β-D-glucose (PGG). PGG is one of the major constituents present in commercial tannic acid with an amount approximately 15%.[17] Several *in vitro* and *in vivo* experiments have shown that PGG has inhibitory activities against several cancers including prostate and breast cancer.[5,30-32] Hence, higher galloyl glucose such as PGG could be the key element that contributes to the cytotoxicity of porcupine bezoars in colon cancer cells.

CONCLUSION

Among three tested porcupine bezoars, crude extract of BD and PD exerted selective toxicity in colon cancer cells but not in normal colon cells. Further, tannin-enriched fractions of both BD and PD induced significantly higher level of cytotoxicity in comparison to that of crude extract indicating hydrolyzable tannins maybe the main bioactive constituent of these porcupine bezoars. At the same concentration, both commercial tannic acid and the tannins-enriched fractions induced apoptotic cell death and cell cycle arrest in HT-29 and HCT-116 cells. In addition, *in vitro* enzymatic test implicates cytotoxic activities of black and PDs may involve inhibition of 5-LOX in human colon cancer cells. Tandem mass spectrometric analysis identified gallic acid and polygalloyl glucoses in the tannin-enriched fractions and these chemical constituents are similar to those identified in commercial tannic acid. Our study suggests that polygalloyl glucoses containing higher number of galloyl groups might be the key to the cytotoxic activity of porcupine bezoars.

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Conflicts of interest

There are no conflicts of interest.

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