Biomedicine & Pharmacotherapy 114 (2019) 108841

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Anticancer activity of grassy *Hystrix brachyura* bezoar and its mechanisms of action: An *in vitro* and *in vivo* based study

Al'aina Yuhainis Firus Khan[a], Qamar Uddin Ahmed[b], Vigneswaran Narayanamurthy[c,d], Shakirah Razali[a], Faizah Abdullah Asuhaimi[a], Mohammed S.M. Saleh[b], Muhammad Farid Johan[e], Alfi Khatib[b], Azman Seeni[f], Ridhwan Abdul Wahab[a,*]

[a] Department of Biomedical Science, Kuliyyah of Allied Health Sciences, International Islamic University Malaysia, 25200, Kuantan, Pahang, Malaysia
[b] Department of Pharmaceutical Chemistry, Kulliyyah of Pharmacy, International Islamic University Malaysia, 25200, Kuantan, Pahang, Malaysia
[c] Faculty of Electrical and Electronics Engineering, University Malaysia Pahang, Pekan, 26600, Pahang, Malaysia
[d] InnoFuTech, No: 42/12, 7th Street, Vallalar Nagar, Pattabiram, Chennai, Tamil Nadu, 600072, India
[e] Department of Haematology, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia
[f] Cluster of Integrative Medicine, Advanced Medical and Dental Institute, Universiti Sains Malaysia, 13200, Bertam, Penang, Malaysia

ARTICLE INFO

Keywords:
Porcupine bezoar
Apoptosis
Cell cycle arrest
Anti-angiogenesis
Anti-metastasis

ABSTRACT

Porcupine bezoar (PB) is a calcified undigested material generally found in porcupine's (*Hystrix brachyura*) gastrointestinal tract. The bezoar is traditionally used in South East Asia and Europe for the treatment of cancer, poisoning, dengue, typhoid, etc. However, limited scientific studies have been performed to verify its anticancer potential to substantiate its traditional claims in the treatment of cancers. Hence, this study was aimed at investigating the *in vitro* and *in vivo* anticancer properties of two grassy PB aqueous extract (PB-A and PB-B) using A375 cancer cell line and zebrafish model, respectively. This paper presents the first report on *in vitro* A375 cell viability assay, apoptosis assay, cell cycle arrest assay, migration assay, invasion assay, qPCR experimental assay and *in vivo* anti-angiogenesis assay using the grassy PBs. Experimental findings revealed IC_{50} value are 26.59 ± 1.37 μg/mL and 30.12 ± 3.25 μg/mL for PB-A and PB-B respectively. PBs showed anti-proliferative activity with no significant cytotoxic effect on normal human dermal fibroblast (NHDF). PBs were also found to induce apoptosis via intrinsic pathway and arrest cell cycle at G2/M phase. Additionally, the findings indicated its ability to debilitate migration and invasion of A375 cells. Further evaluation using embryo zebrafish model revealed LC_{50} = 450.0 ± 2.50 μg/mL and 58.7 ± 5.0 μg/mL for PB-A and PB-B which also exerted anti-angiogenesis effect in zebrafish. Moreover, stearic acid, ursodeoxycholic acid and pregnenolone were identified as possible metabolites that might contribute to the anticancer effect of the both PBs. Overall, this study demonstrated that PB-A and PB-B possess potential *in vitro* and *in vivo* anticancer effects which are elicited through selective cytotoxic effect, induction of apoptosis, inhibition of migration and invasion and anti-angiogenesis. This study provides scientific evidence that the porcupine bezoar do possess anti-cancer efficacy and further justifies its traditional utility. However, more experiments with higher vertebrae models are still warranted to validate its traditional claims as an anticancer agent.

1. Introduction

Natural resources as remedies for medicinal purposes are common in human history. Plant and animals are used as main sources not just for food but also as medicine to cure disease or illness. Moreover, for decades, natural products from plant and animal have been the active ingredient for medicine. In fact 60% of approved anticancer drugs have been isolated or derived from natural resources [1]. Notwithstanding synthetic chemopreventive and anticancer agents are extensively available, drugs obtained from natural products have been reported to demonstrate promising results as well [2]. Presently, extensive research studies have been carried out worldwide to isolate more active and safe bioactive agents including anticancer agents from natural resources including plant, microbes, marine and animals [3,4].

Malignant melanoma is one of the most fatal skin cancers as report in 2016 stated melanoma as 3rd most prevalence cancer in the United State of America for male and 5th for female [5]. Though it can be cured by surgical removal in early stages, once it is developed into

* Corresponding author.
E-mail address: ridhwan@iium.edu.my (R.A. Wahab).

https://doi.org/10.1016/j.biopha.2019.108841
Received 26 December 2018; Received in revised form 26 March 2019; Accepted 31 March 2019

Fig. 1. The bezoars used in this study. The PB with Malaysia 50 sen and scale as size reference and the image of PB after being crushed consists of mixture of rough powder and undigested fibre.a) PB-A, b) PB-B.

metastatic stage, medication becomes extremely difficult as it does not effectively respond to available therapies [6,7]. Melanoma is an extremely aggressive disease with high metastatic potential and a notoriously high resistance to cytotoxic agents. This is thought to be due to the fact that melanocytes originate from highly motile cells that have enhanced survival properties [8]. It is the worst type of skin cancer with increasing incidence compared to other type of cancers [9]. Malignant melanoma consists of four major histological subtypes frequently reported namely superficial spreading melanoma (70%), nodular melanoma (15%), lentigo maligna melanoma (13%), and acral lentiginous melanoma (2–3%) [9]. Additionally, it has highest metastatic potential compared to other skin cancers 10–15% occurring in patients [10]. Furthermore, study revealed patients with metastatic melanoma had poor prognosis with survival rate of 6–8 months only [11]. Malignant melanoma has shown poor response to current therapy with significant toxicity and survival depending on types of treatment [5,6]. Thus, the urgency to find a new alternative for melanoma illness countermeasure is compelling.

In this study, bezoar a natural product obtained from *Hystrix brachyura* (porcupine) is revealed as potential anticancer agent. Porcupine bezoars (phytobezoars) are concretions of undigested plants material that accumulate and calcified in the form of stone within the gastrointestinal tract of porcupine [12]. The word bezoar itself origins in Persian (pad = to expel, zahr = poison) or antidotes [13]. The earliest documented usage of porcupine bezoar has been mentioned in the materia medica of Abu Mansur Muwaffak in the tenth century [13]. The Arabic physicians and crusaders spread it to Europe and other countries as well and by twelfth centuries the bezoars had become widespread use to treat various diseases in western countries [14]. Further literature showed that bezoar had been considered as luxury medicinal item in South East Asian and Europe in 17th century [13,15,16]. The porcupine bezoar used to be frequently presented as gift to royalties and aristocrats due to its rarity and medicinal values [15]. Bezoar is traditionally believed to possess various medicinal benefits [13,17]. Traditionally, people have been using bezoars to treat deadly diseases such as cholera, plague, small pox, measles as well as antidotes for various poisons [12,17,18].

There are reported ancient manuscripts mentioning that bezoars can be used as an antidotes to counteract various deadly diseases and has been used as an antidotes for the diseases and medical illness. Thus, we attempted to evaluate whether PB has similar antidotes properties for cancer. Hence, the aim of this current research was to investigate the anticancer activity of porcupine bezoars on the most malignant melanoma and further furnish scientific proof for its mere medicinal assertion as an anticancer agent.

2. Materials and methods

2.1. Chemical and materials

Human melanoma and normal human dermal fibroblast were obtained from American Type Culture Collection (ATCC), USA. Cells were grown in complete growth medium (CGM) which was made of Dulbecco's modified Eagle medium (DMEM) (Nacalei Tesque, Japan) supplemented with 10% Fetal Bovine Serum (Nacalei Tesque, Japan) & 1% of Penicillin-Streptomycin (Nacalei Tesque, Japan). Phosphate buffer saline (PBS) (Gibco, USA) was used for cells washing. For analysing apoptosis and cell cycle, Nexin reagent (Merck Millipore, USA) and cell cycle reagent (Merck Millipore, USA) were used, respectively. As for invasion assay, matrigel (CORNING, USA) and crystal violet (Sigma-Aldrich, USA) were used. For the mRNA expression analysis, InnuPREP DNA/RNA mini kit (Analytik Jena, Germany), SensiFAST cDNA synthesis kit (BIOLINE, UK), SensiFAST SYBR® No-ROX Kit (BIOLINE, USA) and primer (Integrated DNA technologies, Singapore) were used. 5-Fluorouracil (5-FU) (Sigma, USA), a standard anticancer drug was used as a positive control. The 3,4- dichloroaniline (3,4-DCA)

(Sigma, USA), a standard toxic drug was used as a positive control for embryotoxicity. Paraformaldehyde,methanol, acetone, Tris-base, sodium chloride (NaCl), Magnesium chloride (MgCl), Nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Merck, USA. Tween-20 (ThermoFisher scientific,US).

2.2. Porcupine bezoar extracts preparation

Hystrix brachyura (Porcupine) is one of the protected animals according to the Malaysian law. Hence, prior to the commencement of research study, permission was obtained from the Malaysian government authority namley, Department of Wildlife and National Parks, Malaysia for academic and research purposes (JPHL&TN (IP): 100-34/1.24 Jld 8). The bezoars used in this study are depicted in Fig. 1. The bezoar colour is mixture of dark green and dark brown, the crushed bezoars texture are mixture of powder and grass fibre looks. Initially, the dried grassy porcupine bezoar (PB) (1.09 g) and (8.65 g) were crushed into powdered form using mortar and pestle. Subsequently, 500 mg of powdered grassy PB was extracted using deionized water with 1:20 ratio for 30 min using ultra sonication method to get PB aqueous extract [19]. The PB aqueous extract were filtered and dried in an oven at 40 °C to obtain the extract in powdered form. The final weight of PB aqueous extract are PB-A (125 mg, 25%) and PB-B (215 mg, 45%). The dry powdered form of PB aqueous extracts were then prepared into stock solution (2.0 mg/mL) and further diluted with CGM or DMEM accordingly. On the other hand, the positive control (5-FU) was prepared by dissolving in DMSO, before making it into stock solution (1.0 mg/mL) with CGM and diluted further accordingly. Using PB-A and PB-B aqueous extract, investigation were carried out to observe PB compound profile and anticancer effects on malignant melanoma (A375).

2.3. Cell culture maintenance

A375 and NHDF cells were cultured in a CGM supplemented with DMEM containing 10% Fetal Bovine Serum & 1% of penicillin streptomycin antibiotic. These cells were maintained at 37 °C in 5% CO_2 humidified atmosphere.

2.4. Cell viability assay

This assay was carried out to screen the PB aqueous extracts cytotoxic effect on A375 and NHDF cell lines. Cell viability assay was done to determine 50% inhibitory concentration (IC_{50}), proliferation assay and toxicity assay were evaluated by following Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation assay as recommended by the manufacture. In determining IC_{50}, optimized seeding density of 4×10^2 cells/mL were treated with PB-A and PB-B at different concentrations (3.9–1000 µg/mL) in 96-well plate and incubated for 72 h. The colorimetric assay was measured at 490 nm using a microplate reader (infinite®Pro200 TECAN Group Ltd, Switzerland). The IC_{50} values of both PBs and 5-FU were calculated using Graphpad Prism 6. Calculated IC_{50} of PB-A and PB-B values were used to test toxicity on NHDF and A375 proliferation at 24, 48, 72 and 96 h. Both assays used MTS calorimetric assay. Untreated (UT) cells were used as negative control and 5-Fluorouracil was used as a positive control.

2.5. Cell cycle arrest assay

Cell cycle assay was performed in order to determine whether both PB aqueous extract may induce cell arrest however, through which phase. Grown cells were treated with PB-A and PB-B at IC_{50} for 72 h. All cells from the well were collected and prepared in triplicate. The cells were fixed with cold ethanol for 2 h and stained with cell cycle kit. Cells were analyzed using guava flow cytometer.

2.6. Apoptosis assay

Apoptosis Annexin V/7AAD assay was performed to determine the apoptosis distribution. A375 cells were treated with IC_{50} of PB-A and PB-B for 72 h. All cells from the well were harvested and prepared in triplicate. For apoptosis analyses, cells were stained using Nexin reagent (Millipore, USA) and analyzed using guava flow cytometer (Merck Millipore, USA). Distribution of early and late apoptotic cells after exposure with both PBs was reported in dot plot graph.

2.7. Migration assay

The 2D migration assay was performed to determine the suppressive effect of PB-A and PB-B on migration of melanoma cells. A375 cells were seeded in a 6-well plate for 24 h as described previously by Liang et al. [20]. Once cells got attached, cells were scratched with 100 mL pipette tip and washed with PBS to remove non-adherent cells. Both PBs, 5-FU and media for UT were added in each well accordingly. Photomicrographs of migrated A375 were captured in using an inverted microscope and the distance of cells closure was analysed using Image J software.

2.8. Invasion assay

The aim of invasion assay was to evaluate the ability of PB-A and PB-B in inhibiting A375 cells from invading the matrigel layer and migrate passing through the inserts to lower chamber. Cell invasion assay of A375 was done according to the manufacturer's instructions and as described previously by Berens et al. [21]. Matrigel was diluted into 0.2 mg/mL and pre-coated inserts with 8 µm pores. A375 cells were serum starved for 6 h before trypsinized and seeded (2.0×10^3 cells/well) in upper chamber of inserts. Both PBs dissolved in serum free media was added into upper chamber. Media with 2% of serum was used as chemoattractant in lower chamber. Once 18 h of incubation was achieved, cells which had invaded matrigel were fixed with 4% paraformaldehde and stained with 0.2% crystal violet. Photomicrographs of A375 treated with PB-A, PB-B, 5-FU, UT and uncoated were taken using inverted microscope. Cells were manually counted in 6 random photomicrographs to calculate the percentage of invaded cells.

$$\text{Invasion percentage} = \frac{\text{Number of cells invaded matrigel through insert}}{\text{Number of cells migrated through uncoated insert}} \times 100\%$$

2.9. Real time quantitative PCR (RT-qPCR) analysis

The aim of this assay was to quantify the mRNA expression of mRNA of targeted primers related to apoptosis, cell cycle arrest and metastasis. Total RNA was extracted from PB-A and PB-B treated or untreated A375 cells after 72 h using RNA extraction kit according to manufacturer's instructions. The RNA's quality and integrity were assessed before quantification process. The complementary DNA (cDNA) was prepared using cDNA synthesis kit according to manufacturer's instructions. 200 ng of RNA template was synthesized into cDNA and later diluted in the experiments. The primers were chosen from National Center for Biotechnology Information (NCBI) database with a melting temperature (Tm) of 59–65 °C. Amplicon size was 70–150 bases. Forward and reverse primers spanning exon-exon junctions were selected to avoid amplification of genome sequences. PCR amplifications were performed using SYBR green in CFX96 Touch™ Real-Time PCR. Annealing temperature was optimized using gradient temperature setting. Melting curves were analysed to ensure amplification specificity and null primer-dimer formation. Primer PCR efficiency was evaluated using serial dilutions of cDNA sample (1:10, 1:100, 1;1,000, 1:10,000 and 1:100,000) in CFX Manager software. The amplification efficiency (E)

and correlation coefficients (r^2) of the standard curve ranging from 93.0% to 114.7% and 0.984 to 0.998 respectively. Details information about the primers is attached in supplimentary 1. The amplifications were done by following the conditions viz., 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Results were analyzed using CFX Manager Software based on the threshold cycle (CT) values. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB) were used as reference genes. The genes used and the nucleotide sequences are summarized in supplementary 1.

2.10. Zebrafish husbandry

Zebrafish were bred and maintained in the Centre of Research and Animal Management, International Islamic University Malaysia, Malaysia. The zebrafish were maintained at 28 °C with 14 h/10 h light/dark cycle. The spawning took place with ratio of 4:2 male to female. The fertilized embryos were collected at 3 hpf, cleaned with egg water (0.2 g/L ocean salt in distilled water with 1 ppm methylene blue).

2.10.1. Zebrafish toxicity evaluation

The aim of zebrafish toxicity assay was to evaluate PB-A and PB-B effect on the median lethal concentration (LC_{50}). The assay were performed according to the fish embryo toxicity test guidelines with slight modifications [22]. Briefly, embryos at 3 hpf were randomly selected and transferred into 300 µL PB-A and PB-B diluted with egg water. The concentration tested was optimized to ensure the range concentation tested induce 100% mortality and 100% alive. The embryos were exposed with PB-A of 0.25, 0.5, 0.75, 1.0 and 1.25 mg/mL while PB-B of 3.9, 7.8, 15.6, 31.25,62.5, 125, and 250 µg/mL for 96 h with egg water as negative control. Twenty embryos were used for each group, and the assay was repeated three times. The mortality was observed and calculated which later analysed with Graphpad Prism software.

2.10.2. Antiangiogenesis assay

The aim of the assay was to evaluate whether PB-A and PB-B could inhibit intersegmental vessels (ISVs) blood vessel formation. The assay was performed as described previously. Briefly, twenty healthy embryos at 24 hpf (21 somite stage) were randomly selected and treated by incubating in embryo water containing PB-A (1.25, 0.75 and 0.25 mg/mL), PB-B (125, 31.25 and 7.8 µg /mL), together with untreated as negative control [23]. After treatment for 72 h of exposure, embryos were returned to normal egg water. Later, embryos were collected and fixed overnight at 4 °C with 4% paraformaldehyde. Then, the embryos were dehydrated with methanol, rinsed with PBST, and equilibrated with NTMT(100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl2, 0.1% Tween- 20). The staining reaction was started by incubating embryos with NBT/BCIP solution for about 15–30 min according to the protocol. After staining was completed, the embryos were washed with PBST. Each embryo images were taken under inverted microscope. The number of ISV for both PBs treated and UT were manually counted. The percentage of ISV inhibition were calculated as describe previously [24]

$$\text{ISV inhibition}\,\% = 1 - \frac{N(\text{PB ISV})}{N(UT\ ISV)} \times 100\%$$

2.11. Gas chromatography mass-spectroscopy (GCMS) profile of porcupine bezoar extracts

There is no reference available in the literature on porcupine bezoar chamical profile, hence the purpose of this assay was to examine the grassy PB-A and PB-B profile. Both PBs were derivatized as described previously by Javadi et al. [25]. GCMS-TQ8030 linked to a GCMS-QP 2010 Plus Shimadzu gas chromatograph equipped with a capillary column DB-5 (0.25 µm thickness × 0.25 mm diameter × 30 mm length) was used for compound identification. Helium (1.0 mL/min) was used as the carrier gas. The instrument was operated in electron impact mode at ionization voltage (70 eV), injector temperature (250 °C), and detector temperature (280 °C). The starting oven temperature was set at 50 °C and held for 5 min. This temperature was then programmed to 150 °C at 5 °C min and held for 5 min. Finally, the temperature was programmed to 300 °C for 5 min at a rate of 5 °C min. The compounds identification from the spectral data was based on the available mass spectral records in NIST database.

2.12. Statistical analysis

All values were presented as mean ± SD of triplicate from three different experiments (n = 3). A one-way analysis of variance (ANOVA) was performed using the Prism statistical software package (GraphPad Software, USA). Differences between PB-A and PB-B treated with UT were considered significant at $p < 0.01$.

3. Results

3.1. Porcupine bezoar extract inhibited cell growth of A375

To evaluate the cytotoxic effect of PBAE on human melanoma, A375 cells and NHDF cells were used in this study. Various concentrations of PB-A and PB-B were treated on A375 cells and analysed using MTS assay. PB-A and PB-B demonstrated a dose dependent inhibitory effect on A375 cells growth. The IC_{50} values of both PB have been reported together with 5-FU in Fig. 2(a). The IC_{50} values of both PBs on human breast cancer cell (MCF-7) and human cervical cancer cell (HeLa) is attached in supplimentary 2.

The cytotoxic effect of IC_{50} of PB-A and PB-B were further analyzed through proliferation assay. The result in Fig. 2 (b) revealed that both PB-A and PB-B inhibits cell growth significantly across time incubation when compared to UT. Using the same IC_{50} value of PB-A and PB-B, cytotoxicity study was conducted on NHDF cells. Fig. 2 (c) displays PB-A and PB-B effect on NHDF after exposure for 72 h. The finding shows PB-A and PB-B showed no significant toxicity effect towards NHDF cells (normal cells). The finding was supported by morphological examination at 72 h of incubation. In Fig. 2 (d), A375 cells treated with the PB-A and PB-B displayed reduced cells density when compared to UT and presence of cells debris. PB-A and PB-B treated on NHDF showed no significant toxic effect as the cells morphology was intact.

3.2. Porcupine bezoar extracts induced cell cycle arrest and apoptosis

Subsequently, the study investigated the connection of A375 cell growth inhibition with cell cycle arrest. A375 cells were exposed with PB aqueous extract treatment for 72 h and analyzed the cell cycle distribution using flow cytometry. As depicted in Fig. 3 (a and b), PB-A and PB-B induced significant cells arrest in G2/M with the evidence of significant decreasing of cells proportion in G1/G0 phase by 36.4%(PB-A), 37.5%(PB-B), increasing of cells proportion in G2/M phase by 50.4%(PB-A), 48.1%(PB-B) compared to UT 44.5% and 37.6%, respectively. Expression of key genes involved in G2/M phase transition was further investigated to elucidate the mechanism using qPCR in Fig. 3 (c). The findings showed that both PBs had significant effect by down-regulating the expression of CDK1 and cyclin B1 relative to control. The mRNA expression indicated that PB-A and PB-B induced G2/M arrest by inhibiting cyclin B1/CDK1 complex. Both PBs shows down-regulating of cyclin B1 0.4 fold, 0.5 fold and CDK1 by 0.37 fold, 0.62 fold for PB-A and PB-B respectively.

Further analysis was carried out to evaluate whether PB-A and PB-B inhibits the cells growth of A375 cells due to apoptotic activity. For this reason, both PBs treated cells were stained with Annexin V/7AAD. As displayed in Fig. 3 (d and e), both PBs induced apoptosis resulting in significant distribution of early apoptosis in PB-A and PB-B treatment for 72 h. Dot plot graph in Fig. 3 (d) demonstrates that PB-A and PB-B induced early apoptosis by 44.31% and 40.51% while for late apoptosis

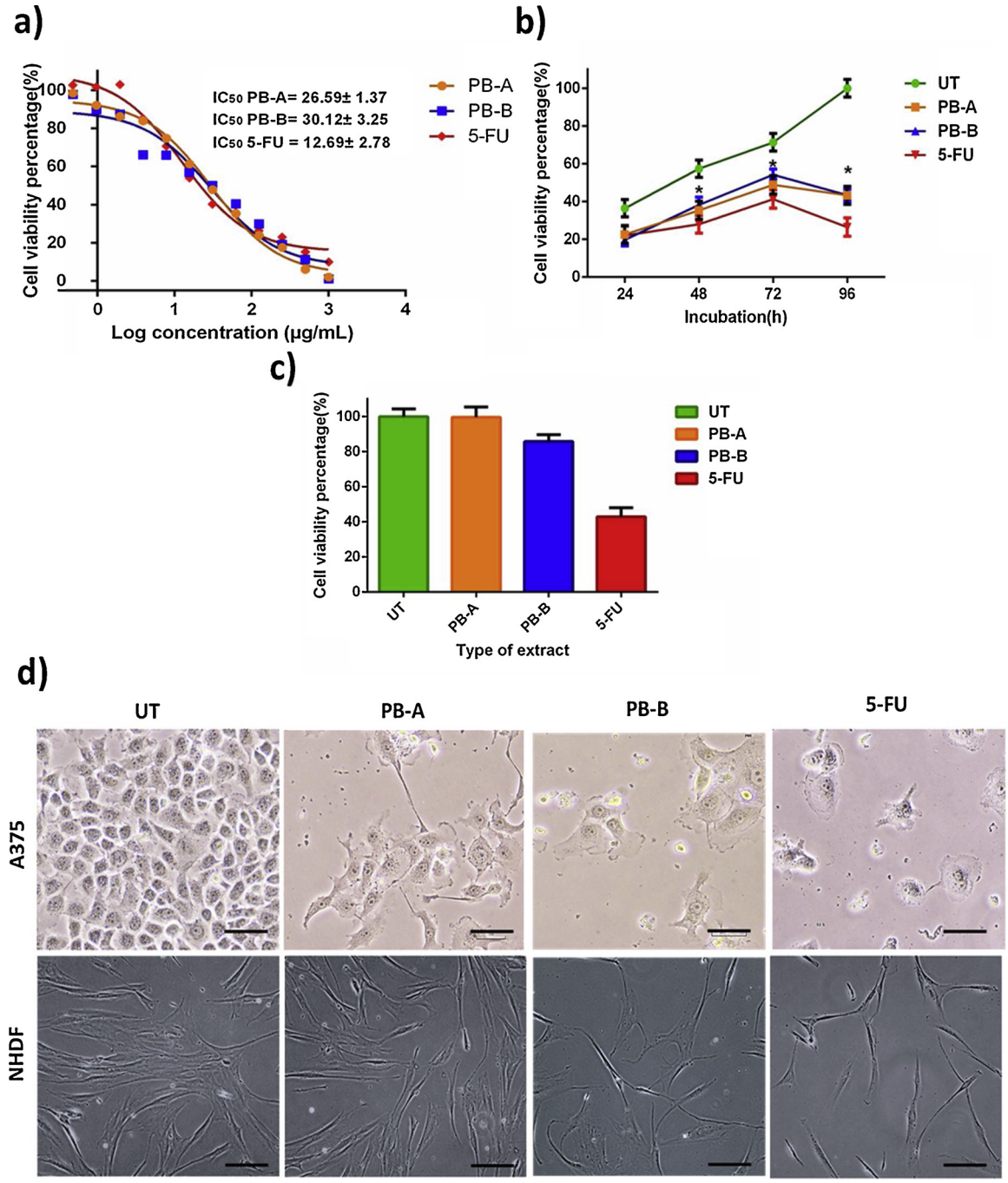

Fig. 2. Effect of PB-A and PB-B on A375 and NHDF cells viability. a) Median concentration of A375 treated with PB-A, PB-B and 5-FU. Graph shown are after the exposure with treatment for 72 h. IC_{50} are presented in mean ± SD (n = 3). b) Anti-proliferative effect of PB-A and PB-B at 24, 28, 72, 96 h with UT and 5-FU as control. c) Cytotoxicity of PB-A and PB-B against NHDF cells at 72 h exposure. Results are presented in mean ± SD (n = 3). d) Image of A375 cells and NHDF upon treated with PB-A, PB-B, UT and 5-FU for 72 h. Bar graph = 15 µm. * indicates $p < 0.01$.

by 4.66% and 10.32% compared to UT which were 3.06% and 0.75%, respectively. Fig. 3 (e) reveals bar graph proportions for PB-A of apoptotic cells (including early and the late apoptosis) and live cells which are 48.5% and 52.5%, while PB-B 50.8 and 49.2 respectively. To understand the mechanism of apoptosis at molecular level, qPCR experiment was performed on key gene viz. *Bax* (pro-apoptosis), *Bcl2* (anti-apoptosis), *cyto C*(cytochrome C family), *cas 3* and *cas 9* (caspases family). Fig. 3 (f) displays PB-A significantly down-regulated Bcl2 (0.3 fold), up-regulation of Bax (1.7 fold), cyto C (3.1 fold), cas 3(4.4 fold) and cas 9 (2.6 fold). PB-B shows similarly effect by down-regulated Bcl2 (0.5 fold), up-regulated Bax by (2.9 fold), cyto c by (3.0 fold), cas 3 by (2.2 fold), and cas 9 by (4.6 fold). The mRNA expression result suggested that both PBs induces apoptosis via mitochondria apoptosis pathway.

3.3. Porcupine bezoar extracts anti metastatic effects on A375

The PB ability to inhibit migration and invasion was evaluated using wound healing assay and transwell membrane assay. A 2D migration assay was followed to determine the effect of PB-A and PB-B on inhibition of A375 cells migration. Results in Fig. 4 (a and b) demonstrate photomicrographic images of A375 cells PB-A, PB-B treated with UT

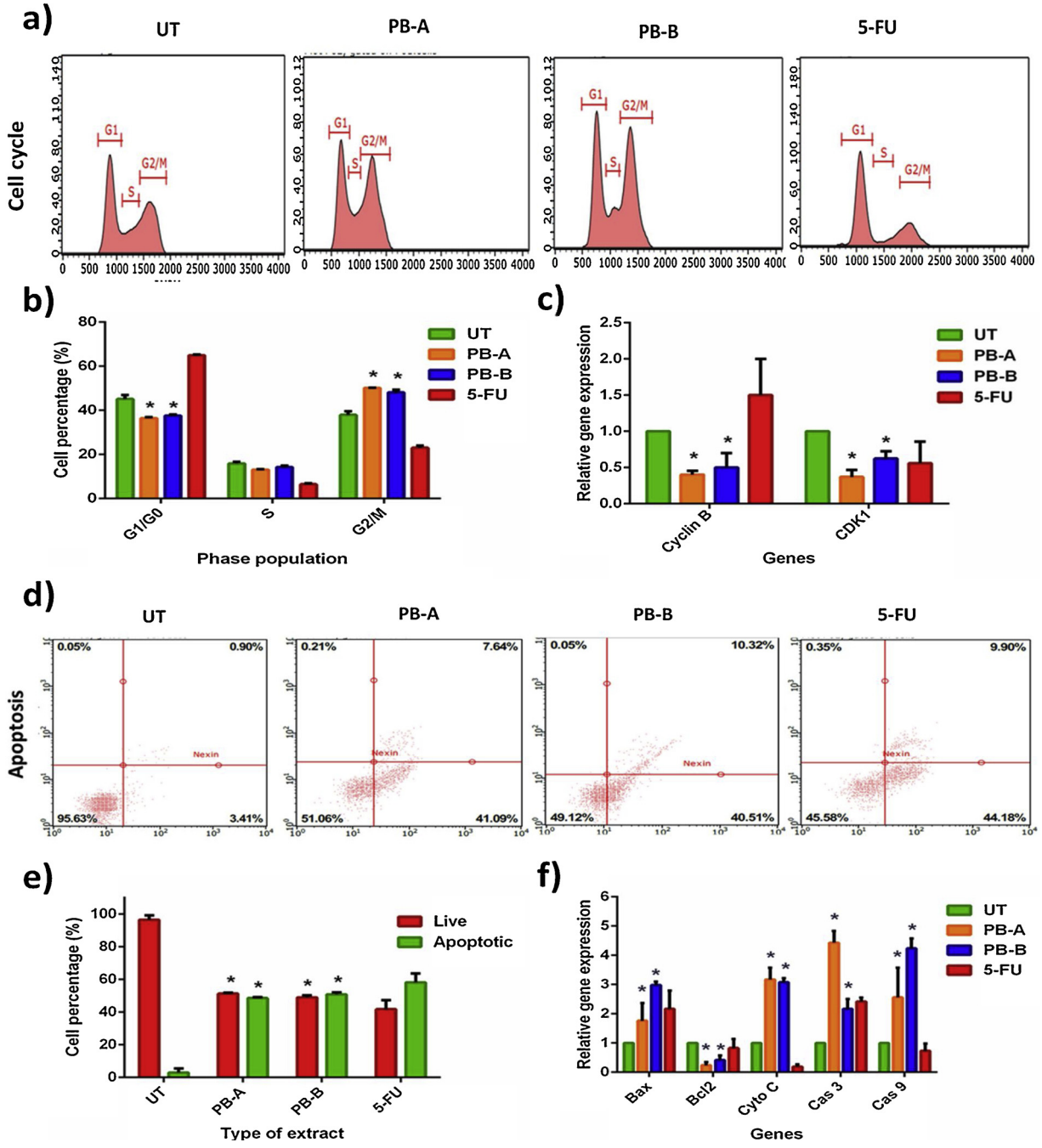

Fig. 3. DNA histogram of PB-A and PB-B treated cells, with UT and 5-FU as control. The analysis was after 72 h of incubation. a) DNA histograms displays cells cycle phases of treated cells namely G1/G0, S, and G2/M. b) The proportion of cells according to cell cycle phase are presented in percentage mean ± SD (n = 3). The modulations of mRNA expressions levels of targeted genes are expressed as relative gene expression based on the calculation using GAPDH and β-actin as the reference gene, assigning the ratio in untreated cells as 1. Data are presented mean ± SD (n = 3). c) The mRNA expression of cell cycle targeted genes genes is presented as relative gene expression mean ± SD (n = 3). d) Dot plot distribution of early and late apoptosis of A375 cells upon exposure with both PBs for 72 h with UT and 5-FU as control. e) The proportion percentage of apoptotic (early and late apoptosis) and live cells are presented as percentage mean ± SD (n = 3). f) The mRNA expression of apoptosis targetted genes is presented as relative gene expression mean ± SD (n = 3). * indicates $p < 0.01$.

and 5-FU as control taken at 24 h. The results in Fig. 4 (b) demonstrate that PB extract effectively inhibited wound closure as percentage cells migrated are 43.2% for PB-A and 57.3% for PB-B when compared to UT which is 85.0%. Similar results were obtained in transwell invasion assay shown in Fig. 4 (a and c). The transwell invasion assay evaluated PB-A and PB-B treated A375 cells to invade the matrigel into lower chamber using 2% FBS as chemoattractant. The finding revealed both PBs inhibited the invasion significantly by invading the matrigel 24.0% (PB-A) and 26.9% (PB-B) compared to UT viz., 75.5%. Gene of matrix metalloproteinases (MMP2 and MMP9), E-cadherin (an important protein in formation of adherens junctions) and NM23 (the metastasis suppressor) were investigated using qPCR. The mRNA expression showed PB-A down-regulated significantly MMP2 by 0.2 fold, MMP9 by 0.3 fold and significantly up-regulated mRNA expression of NM23 and E-cad by 4.5 and 4.1 fold, respectively (Fig. 4 (d)).Additionally PB-B treated cells expressed similarly with PB-A it induce up-regulated in NM23 (2.1 fold), E-cadherin (3.9 fold) and down-regulated MMP2(0.2 fold) and MMP9 (0.3 fold) significantly. The finding suggested that PB-

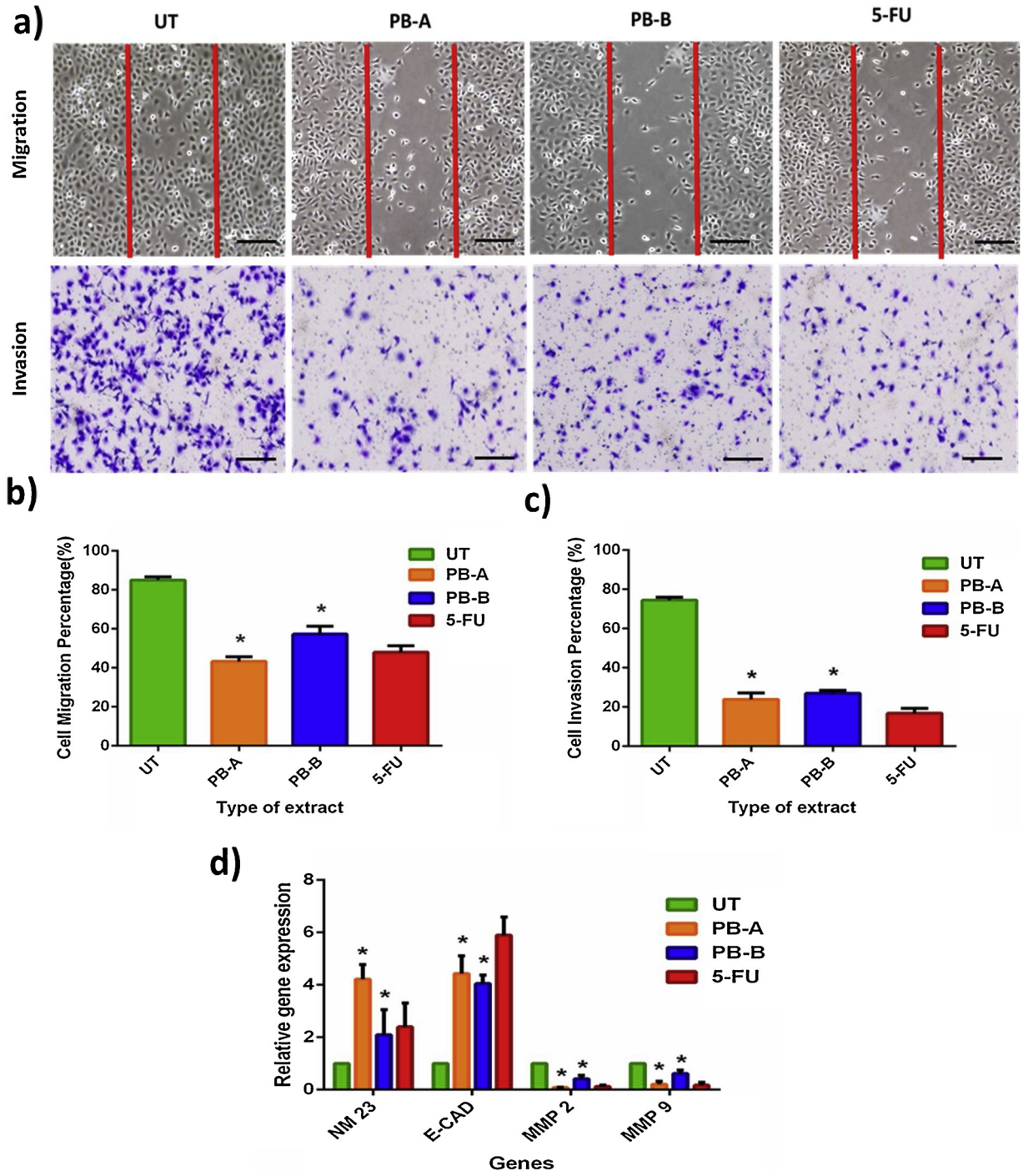

Fig. 4. Anti-metastasis effect of PB-A and PB-B against A375 cells. a) Photomicrographic image of A375 cells upon treatment with PB-A, PB-B, UT and 5-FU were taken at 24 h for migration assay. The images of A375 cells invaded matrigel in transwell membrane to lower chamber with UT and 5-FU as control are hown as well in (a). Bar graph = 25 μm. b) Graphical representation of migration percentage of A375 cells upon both PBs treatment is presented as percentage mean ± SD (n = 3). c) Percentage of A375 invasion upon treatment is presented as percentage mean ± SD (n = 3). The modulations of mRNA expressions levels of targeted genes are expressed as relative gene expression based on the calculation using GAPDH and β-actin as the reference gene, assigning the ratio in untreated cells as 1. d) MRNA expressions of targeted genes related to metastasis are presented as mean ± SD (n = 3). * indicates $p < 0.01$.

A and PB-B inhibits melanoma cells migration and invasion.

3.4. Porcupine bezoars effect on embryotoxicity of zebrafish

This assay was aimed to determine the concentration which may induce 50% mortality to embryos tested at end of the assay with 6 groups of concentration of PB-A, PB-B together with UT as negative control. The finding revealed both PBs exhibited mortality on embryos in dose-dependent manner. The log concentration against probits graph is reported in Fig. 5 (a). The LC_{50} value at 96 hpf were found to be 450.0 ± 2.5 μg/mL and 58.7 ± 5.0 μg/mL for PB-A and PB-B respectively. Additionally, morphology of UT embryo at 96 hpf showed no sign of malformation. Contrary for PB-A, at 1.25 mg/mL, Fig. 5 (b) depicts that PB-A treated embryo showed delayed growth (72 hpf), pericardial edema, yolk sac edema, swim bladder missing, intestine malformation and heart malformation. The embryo treated with

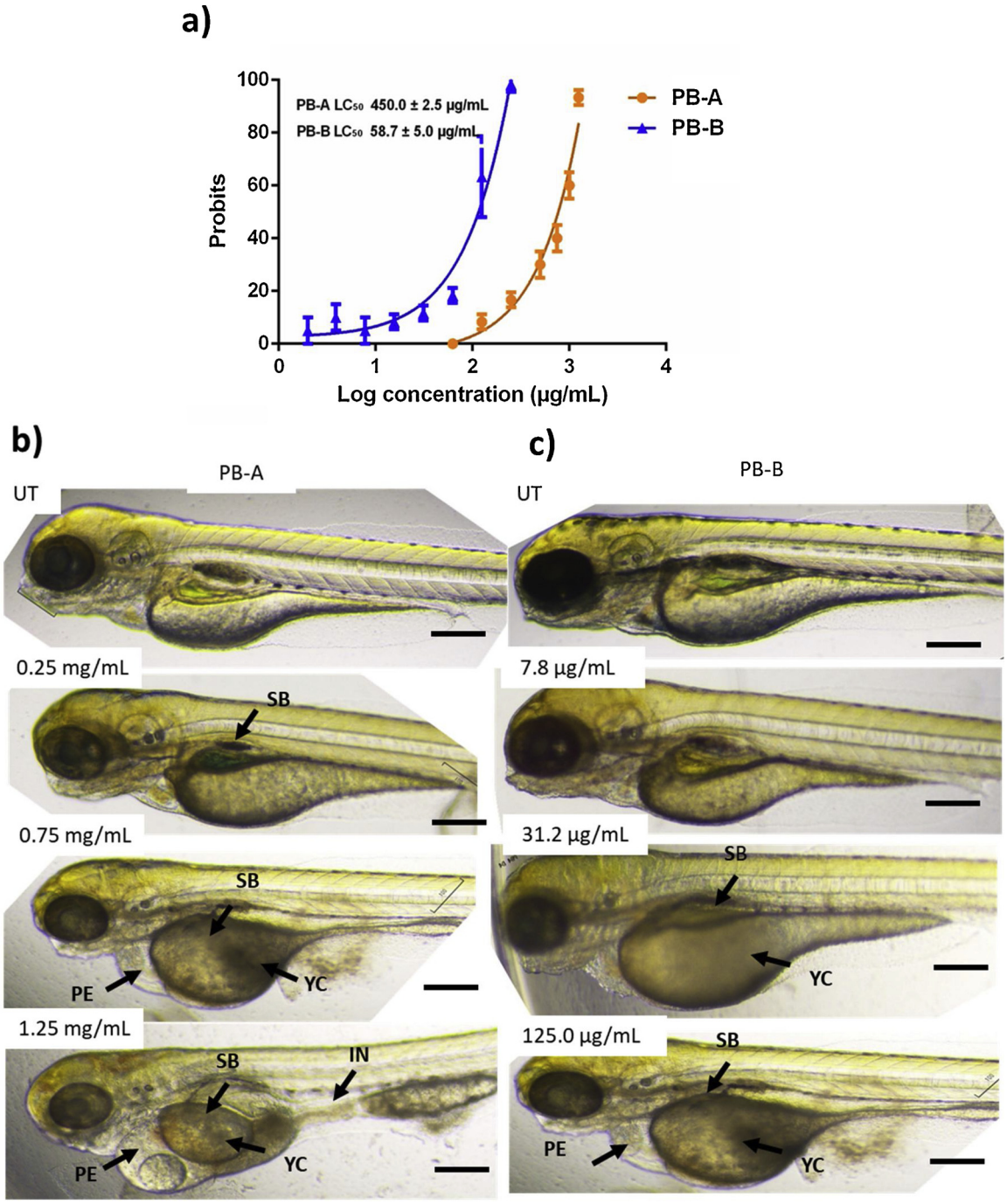

Fig. 5. PB's effect on embryo zebrafish. a)The median lethal concentration of PB-A and PB-B. Assay was performed triplicate with n = 20 for each group, result presented as probit ± SD. b) Representative of PB-A treated embryo for embryotoxicity assay. c) Representative of PB-B treated embryo for embryotoxicity assay. Three concentration was chosen for morphological observation of both PBs treated on embryos with untreated was consider as negative control. Bar Graph = 200 µm. The swimming bladder (SB), yolk sac (YC), pericardial edema (PE) and intestine malformation (IN). Three separate assay was perform with n = 20.

0.75 mg/mL PB-A showed malformation of delayed growth (72 hpf), pericardial edema, yolk sac edema and swim bladder missing. However, the embryo treated with 0.25 mg/mL displayed only slightly delayed growth with malformation of swimming bladder. PB-B was more potent in inducing toxic effect compared to PB-A. The highest concentration observed for toxicity of PB-B was 125.0 µg/mL, where it caused growth delayed, missing swimming bladder, large yolk sac, and slightly pericardial edema. PB-B 31.2 µg/mL induced delayed growth, missing swimming bladder and yolk sac malformation. However, lowest concentration 7.8 µg/mL reported to have only growth delayed without other type of malformation. The finding shows PB-A induced more malformation compared to PB-B.

3.5. Porcupine bezoars effects on angiogenesis of embryo zebrafish

Cancer angiogenesis plays major role in cancer metastasis. Hence, PB-A and PB-B potential as antiangiogenesis agent was further validated through *in vivo* angiogenesis zebrafish model. The treated PBs embryos were evaluated for their effect on ISV formation. Three concentrations of PB-A were used to observe angiogenesis of ISVs viz., 1.25, 0.75 and 0.25 mg/mL.While for PB-B 125.0, 31.2, and 7.8 µg/mL were used. The finding in Fig. 6 (a) and (b) revealed control embryo at the

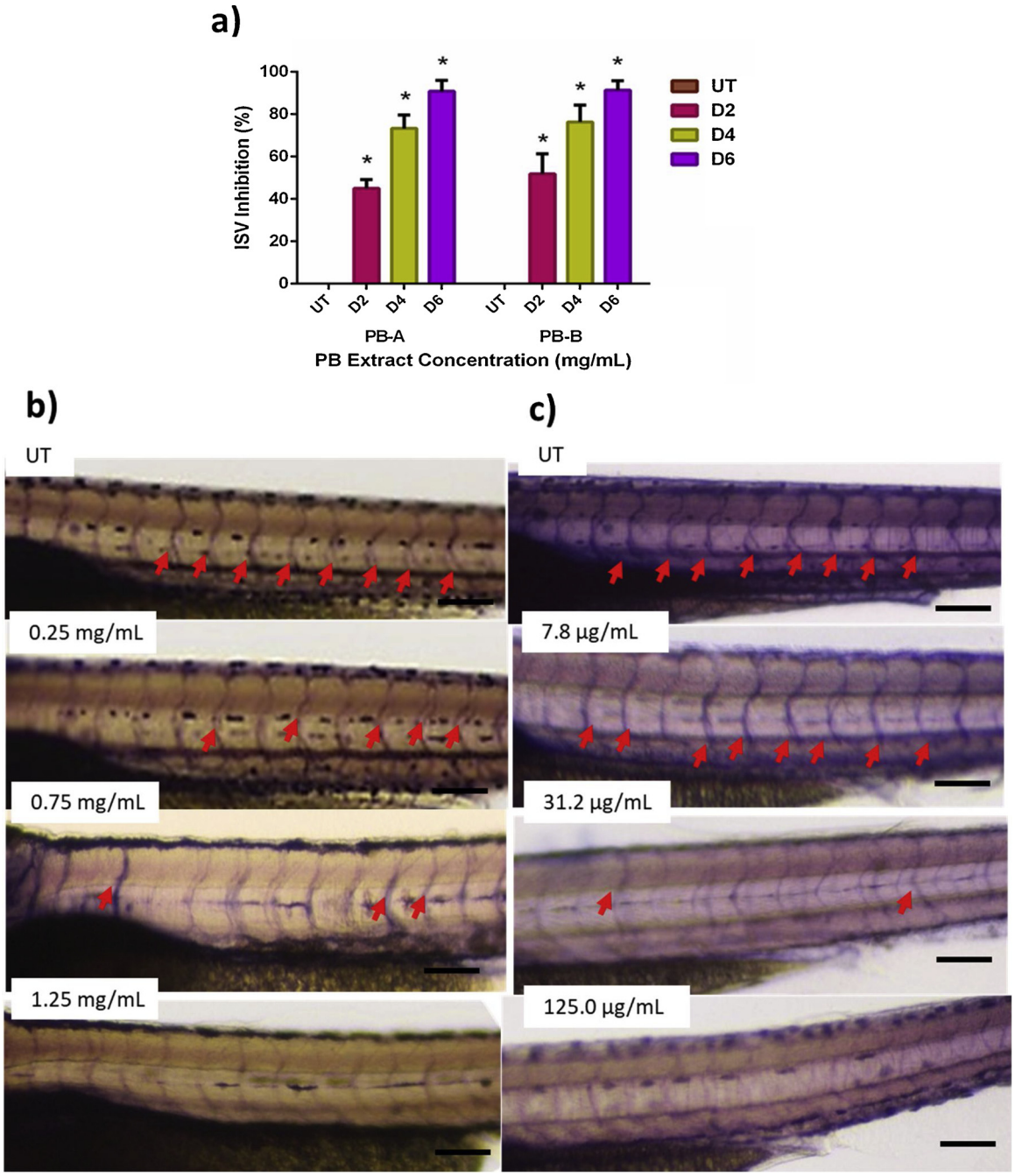

Fig. 6. PB's effect on embryo angiogenesis zebrafish assay. a) Graph representation of ISV inhibition percentage for treated PB-A and PB-B embryos. Assay was performed thrice with n = 20 for each group, result presented as percentage mean ± SD. * indicates $p < 0.01$. b) Representative of angiogenesis assay of PB-A. b) Representative of angiogenesis assay of PB-B. Three concentration viz. 1.25, 0.75, 0.25 mg/mL of PB-A and 125.0, 31.2, 7.8 µg/mL were treated on embryos with untreated considered as negative control. Bar graph = 150 µm. The red arrows indicate complete ISV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

end of assay displayed well sprout ISVs, lumenized with arranging array. On the other hand, PB-A was found to inhibit blood vessel formation in dose dependent manner as it significantly inhibit the vasculature formation by 90.4%, 73.3% and 45.0% at 1.25 mg/mL, 0.75 mg/mL and 0.25 mg/mL, respectively. Similar effect found in PB-B where it found to inhibit vasculature formation by 91.4%, 76.4% and 9.5% at 125.0 7.8 µg/mL, 31.2 7.8 µg/mL, and 7.8 µg/mL respectively.

3.6. Porcupine bezoars aqueous extract GC–MS chemical profile

The chromatogram of PB-A and PB-B is shown in Fig. 7 and Table 1 presents the compound for each major peak in the chromatogram. Table 1 displays both PB aqueous extract's compounds with their, molecular formula, peak percentage and probability index. The GC–MS analyses of PB-A revealed major components namely dilauryl thiodipropionate (47.27%), pentadecyl acrylate (10.36%), 5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1′,2′-d]pyrazine (9.11%) and lauryl 3-mercaptopropionate (7.24%). On the other hand, PB-B major components revealed 5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1′,2′-d]pyrazine (11.28%), Ursodeoxycholic acid (8.63%), Cholest-5-en-3-ol (3.beta.)-, carbonochloridate (7.25%), Pentadecyl acrylate (4.10%) and Stearic acid (3.50%). The compounds

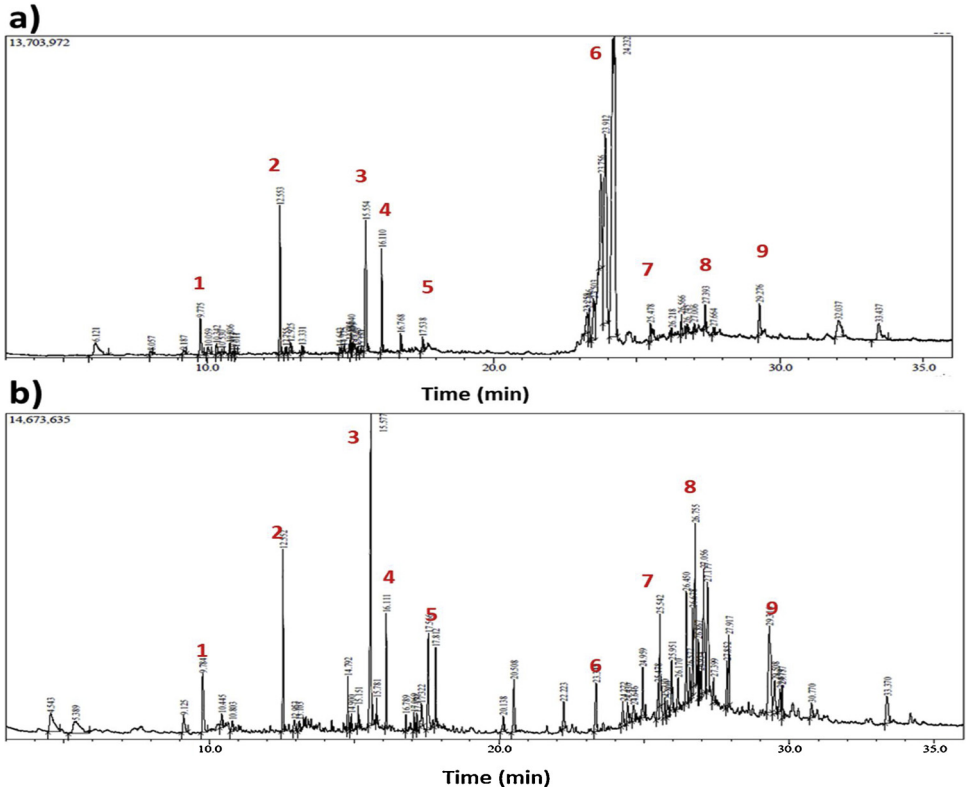

Fig. 7. The chromatogram of GC–MS profile for both PBs used in this study. a)PB-A and b) PB-B.

structures and mass spectrum for each compound are reported in a supplimentary 3 file.

4. Discussion

Melanoma is one of the deadliest malignant skin cancers with high incidence and drugs resistance. Therefore, potential anticancer agents with minimal toxicity are considered promising candidates. In this study, two PB aqueous extract namely PB-A and PB-B were assessed for its *in vitro* and *in vivo* potency as an anticancer agent against A375 cells and zebrafish, respectively. The median inhibition concentration assay revealed low concentrations for both PB needed to inhibit 50% of A375 cells growth at 72 h. However, further analysis demonstrated that both PB exhibits cytotoxic and cytostatic capacity in a dose and time dependent manner. Challenge with chemotherapy and other current drugs despite the ability to kill cancer cells also is to kill normal cells surrounding the cancer as well [11,26]. Hence, cytotoxicity on normal cell is an important aspect of this study which sought to determine the effect of PB-A and PB-B on normal cell lines (NHDF). The assay revealed that PB-A and PB-B did not pose any significant toxicity towards NHDF compared to 5-FU which is a standard anticancer agent. Additionally, the morphology of both PBs treated cells showed apoptosis signs comprising cells shrinkage, cytoplasm rounding, presence of cells debris and reduction in cells viability numbers which are hallmark of apoptosis [27].

Cells cycle analysis was conducted to understand the mechanism of anti-proliferation effect of PBs. The results indicated that PB-A and PB-B arrested G2/M phase in A375 by down regulating mRNA expression of cyclin B1 and CDK1 which are crucial in forming cyclin B1/CDK 1 complexes. The inhibition resulted in preventing A375 cells from entering mitosis phase and arrested in G2 phase for either recovery or apoptosis [28]. However 5-FU in this study reported to arrest A375 cells in G1/S phase. Previous study reported similar study where 5-FU arrest Human laryngeal squamous cancer cells(UMSCC11 A and UMSCC12) by increasing retinoblastoma (pRb) tumor suppressor protein and decreasing cyclin E expression [29]. Furthermore, the ability of PBs to induce apoptosis was evaluated using apoptosis Annexin V/AAD assay. In analysing the apoptosis, the cells were categorised in quadrants base in the presence or absence of phosphatidylserine and 7-AAD detection which help in differentiating early, late apoptotic and necrotic cells [30]. The finding showed both PBs induced apoptosis when compared to UT cells and 5-FU treated cells. The underlying mechanism of

Table 1
GC–MS spectral analysis of compound presents in both PB-A and PB-B.

Peak	Compound	Retention time	Molecular formula	Peak (%) PB-A	Peak (%) PB-B	Probability Index
1	1-Dodecanol	9.755	$C_{12}H_{26}O$	2.54	2.91	97
2	Pentadecyl acrylate	12.53	$C_{18}H_{34}O_2$	10.36	4.10	91
3	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine	15.554	$C_{14}H_{22}N_2O_2$	9.11	11.28	91
4	Lauryl 3-mercaptopropionate	16.110	$C_{15}H_{30}O_2S$	7.24	2.32	93
5	Stearic acid	17.812	$C_{18}H_{36}O_2$	2.52	3.50	90
6	Dilauryl thiodipropionate	23.258	$C_{30}H_{58}O_4S$	47.27	2.56	78
7	Pregnenolone	25.542	$C_{21}H_{32}O_2$	1.25	2.73	85
8	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate	29.276	$C_{28}H_{45}ClO_2$	2.21	7.25	80
9	Ursodeoxycholic acid	33.437	$C_{24}H_{40}O_4$	2.37	8.63	83

apoptosis induction was investigated at molecular level using qPCR analysis. The mRNA expressions of PBs treated indicated apoptosis occurs via mitochondria apoptosis pathway. The intrinsic pathway is initiated within cells involving the Bcl2 families such as pro-apoptosis (Bax) and anti-apoptosis (Bcl2) genes to regulate the promotion or inhibition of apoptosis [31]. PBs also found to up-regulate the expressions of cytochrome C, caspase 9 and caspase 3. The finding was found to be in line with intrinsic pathway in which internal stimuli induce bax to activate cytochrome C release into cytosol and activate caspase cascade through caspase 9 and caspase 3 [32–34]. Additionally, it has been reported that 5-FU induces apoptosis through perforin/granzyme pathway which is different from PB pathway, hence, explains the mRNA expression which is contrary to PBs mRNA expression [35]. Another study reported 5-FU induced its anticancer effects by inhibiting thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA which lead to DNA damage [36].

Although there are many anticancer drugs available to inhibit cancer growth, less number of drugs available to inhibit metastasis of cancer [37]. Melanoma is highly invasive and metastatic in nature, hence in this study, the migration and invasion of PB-A and PB-B treated A375 cells were evaluated. Metastasis consists of multi-step process involving adhesion of cells, invasion and migration [38]. Therefore, intervention in any of the steps is considered the key factor to inhibit metastasis progression. The finding suggested for both PBs to be able to suppress A375 cells migration and matrigel invasion hence signifies its potential as an anti-metastasis agent. Matrix metalloproteinases (MMPs) play roles in degradation of extracellular matrix (ECM) components and the MMPs which contribute in metastasis are MMP2 and MMP9 [39]. Thus down-regulation of MMP 2 and MMP 9 indicates inhibition degradation of ECM. Additionally, E-cadherins up regulation indicated invasion was hinder as cadherins play important roles in cell adhesion where it knits together at the intercellular junction through dissociating from its neighbour. Hence increasing E-cadherin genes, showed A375 invasion inhibited [40]. NM23 is also known as metastasis suppressor which play a key role in metastasis, hence up regulation of NM23 genes in PB-A and PB-B treated cells suggested that A375 cells were suppressed from migrated and invaded [41,42].

The embryo zebrafish model was considered the most suitable animal model in this study as it mimics the physiology of mammals, rapid, fast and efficient screening with less usage of [42–44]. The embryo toxicity assay revealed PB-A exerted significant toxicity on embryos only at high concentration, whereas at low concentration PB-A exerted delayed growth. However, PB-B LC_{50} exert significant toxicity effects at low concentration, indicating PB-B was more toxic compared to PB-B. This finding is crucial as people generally consume PB by infusing it into water for hours until it tastes bitter or sometimes take certain amount orally without even realising about the dose taken and its toxicity effect. Additionally, it is revealed each PB has different level of toxicity level. Hence, further evaluation should be done to understand the toxicity effects of bezoar. The angiogenesis is an essential phase for tumor progression and spreading to other parts of body [45]. Hence, this study investigates PBs effect on antiangiogenesis *in vivo* to further confirm its anticancer potential. The angiogenesis assay using embryo zebrafish is considered the feasible animal model as conventional *in vitro* assay lacks the biological complexity of blood vessels and common *in vivo* model such as chick chorioallantoic membrane assay requires a large quantity of extract are the major drawback for PB extract [44,46]. The PB-A extracts revealed to have antiangiogenic potential at all concentrations tested even at 250 μg/mL which is lower than the LC_{50} reported in this study. PB-B as expected shown anti-angiogenic effects as well at all concentration but the assay revealed PB-A shown to be more potent in exerting the antiangiogenic effects as at 31.25 μg/mL it inhibit more than 76.4% which is more lower than the LC_{50} of PB-B.Moreover, it can be deduced that the antiangiogenic effect also affects the growth of the embryos which further explains the growth delayed in the embryo toxicity assay.

Gas chromatography-mass spectrometry analysis was performed to determine the chemical profile of PB-A and PB-B. Compounds identified were present in both PB-A and PB-B. Previous study reported stearic acid and ursodeoxycholic acid compounds possessed an anticancer activity [47,48]. Additionally stearic acid and ursodeoxycholic acid compounds are found to be major compound in calculus bovis (cow bezoar) as well [49]. Stearic acid is found primarily in animal derivatives and plant fat where it is mainly used as dietary and cosmetics [50–52]. Stearic acid reported to induce apoptosis in MDA-MB-231 through mitochondria pathway by reducing mitochondria membrane potential [53,54]. Ursodeoxycholic acid is a synthetic bile acid which reported used for treatment of gallstones and primary biliary cirrhosis [55]. Additionally the ursodeoxycholic acid reported exhibited anticancer properties by inhibit cells proliferation through regulation of oxidative stress in colon cancer cells [48,56]. Moreover, ursodeoxycholic acid had high capacity to scavenge hydroxyl and lipid peroxidation antioxidant assays [57]. Another compound detected in both PBs is pregnenolone. Pregnenolone is a precursor for the synthesis of corticoids and androgens, and it displays neuroactive properties [58]. Pregnenolone revealed to had anti-cancer activity against hepatocellular carcinoma cell line (HepG2) by inducing apoptosis through DNA fragmentation [59]. Furthermore among detected compounds, dilauryl thiodipropionate has been reported to be used as food additive for its secondary antioxidant properties in the food industry [60]. Previous study reported pentadecyl acrylate, have DPPH and ABTS antioxidant activity [61]. However remaining compounds have no previous study related to any biological activity.

The bezoars are formed from reaction of undigested porcupine food and enzymes from porcupine itself. Additionally, it is crucial to understand that each PB is unique and comes from different porcupine. Moreover, the food habbit of individual porcupine might also differ from one another that may also contribute for diversed chemical profile among bezoars. Furthermore, the age of the bezoar in porcupine and location of the bezoar collected could also play major roles for the different chemical profile and quantity of the compounds in bezoars. Hence, support strong possibility of varried biological effects amongst bezoars obtained from different porcupines.

5. Conclusion

The findings in this study are the first to report two PB aqueous extract (PB-A and PB-B) exhibits anti-melanoma properties. Both PBs aqueous extract showed that it could inhibit cells migration, invasion and angiogenesis possibly through cell growth inhibition, arrest cell cycle in G2 phase and induction of apoptosis via mitochondria pathway. Most importantly, PB-A and PB-B extract possesses anticancer properties without significant toxicity on normal cells. Therefore, this study suggests that PB is a potential agent for inhibiting melanoma growth and metastasis.The GCMS profile revealed PB-A and PB-B have same compound presents in both PB. Stearic acid, ursodeoxycholic acid and pregnenolone compounds may be responsible for the anticancer effect of PB extract. However, other unidentified compounds which were not present in the library of the GC–MS system could not be identified from their accurate mass, may also play some roles towards its anticancer effect. Hence, further research is still needed to obtain such bioactive compounds in pure form for complete pharmacological evaluations as anticancer agents. Moreover, further analysis involving higher vertebrae *in vivo* models should also be done to understand the PB effects in living organism more meticulously as well as its identified compounds as anticancer agents.

Conflict of interest

Authors declare, there is no conflict of interest.

Acknowledgments

Authors would also like to thank and acknowledge the Ministry of Higher Education (MOHE), Malaysia for funding this research to accomplish through FRGS 13-055-0296 and FRGS 16-045-0544. Special thanks to the Department of Wildlife and National Parks Malaysia for granting an approval to conduct this study at IIUM. PDF scholarship confererred to V.N by University Malaysia Pahang is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.108841.

References

[1] A. Malik, S. Abdul, M. Universiti, S. Sabbar, D. Universiti, L. Elsir, A. Universiti, In vitro anti-metastatic and antioxidant activity of Nicotiana glauca fraction against breast cancer cells, Adv. Biol. Res. (Rennes) 9 (2015) 95–102, https://doi.org/10.5829/idosi.abr.2015.9.2.9521.

[2] B.B. Aggarwal, S. Shishodia, Molecular targets of dietary agents for prevention and therapy of cancer, Biochem. Pharmacol. 71 (2006) 1397–1421, https://doi.org/10.1016/j.bcp.2006.02.009.

[3] L. Wang, C. Dong, X. Li, W. Han, X. Su, Anticancer potential of bioactive peptides from animal sources (Review), Oncol. Rep. 38 (2017) 637–651, https://doi.org/10.3892/or.2017.5778.

[4] A. Bhanot, R. Sharma, M.N. Noolvi, Natural sources as potential anti-cancer agents: a review, Int. J. Phytomed. 3 (2011) 09–26, https://doi.org/10.5138/ijpm.v3i1.278.

[5] K.D. Miller, R.L. Siegel, C.C. Lin, A.B. Mariotto, J.L. Kramer, J.H. Rowland, K.D. Stein, R. Alteri, A. Jemal, Cancer treatment and survivorship statistics, 2016, CA Cancer J. Clin. 66 (2016) 271–289, https://doi.org/10.3322/caac.21349.

[6] V. Gray-schopfer, C. Wellbrock, R. Marais, Melanoma biology and new targeted therapy, Nature 445 (2007) 851–857, https://doi.org/10.1038/nature05661.

[7] T. Qiu, H. Wang, Y. Wang, Y. Zhang, Q. Hui, K. Tao, ScienceDirect Identification of genes associated with melanoma metastasis, Kaohsiung J. Med. Sci. 31 (2015) 553–561, https://doi.org/10.1016/j.kjms.2015.10.002.

[8] V. Gray-schopfer, C. Wellbrock, R. Marais, Melanoma biology and new targeted therapy, Nature 445 (2015) 851–857, https://doi.org/10.1038/nature05661.

[9] V. Narayanamurthy, P. Padmapriya, A. Noorasafrin, B. Pooja, K. Hema, A.Y. Firus Khan, K. Nithyakalyani, F. Samsuri, Skin cancer detection using non-invasive techniques, RSC Adv. 8 (2018) 28095–28130, https://doi.org/10.1039/c8ra04164d.

[10] R. Chakraborty, N.C. Wieland, I.N. Comfere, Molecular targeted therapies in metastatic melanoma, Pharmgenomics Pers. Med. 6 (2013) 49–56, https://doi.org/10.2147/PGPM.S44800.

[11] F. Tas, Metastatic behavior in melanoma: timing, pattern, survival, and influencing factors, J. Oncol. 2012 (2012) 1–9, https://doi.org/10.1155/2012/647684.

[12] E. Mori, A. Sforzi, Structure of phytobezoars found in the stomach of a crested porcupine, Hystrix cristata L., 1758, Folia Zool. 62 (2013) 232–234, https://doi.org/10.25225/fozo.v62.i3.a9.2013.

[13] C.J. Duffin, Porcupine stones, Pharm. Hist. (Lond.) 43 (2013) 13–22.

[14] M. Barroso, Bezoar stones, magic, science and art, Geol. Soc. Lond. (2013) 1–16, https://doi.org/10.1144/SP375.11.

[15] L. Beaven, K.J. Lloyd, The evidence of the 1698 death inventory, Part ii, Cardinal Paluzzo Paluzzi degli Albertoni Altieri and his collection in the Palazzo Altieri, J. Hist. Collect. (2018) 1–16, https://doi.org/10.1093/jhc/fhx057.

[16] S.H. Smith, The mystification of spices in the western tradition, Eur. Rev. Hist. Rev. Eur d' Hist. 8 (2001) 37–41, https://doi.org/10.1080/13507480120074233.

[17] M.D.S. Barroso, The bezoar stone: a princely antidote, the távora sequeira pinto collection – oporto, Acta Med. Hist. Adriat. 12 (2014) 77–98.

[18] M. Stephenson, From marvelous antidote to the poison of idolatry: the transatlantic role of andean bezoar stones during the late sixteenth and early seventeeth centuries, Hisp. Am. Hist. Rev. 90 (2014) 1–39, https://doi.org/10.1215/00182168-2009-089.

[19] M. Rezaie, R. Farhoosh, M. Iranshahi, A. Sharif, S. Golmohamadzadeh, Ultrasonic-assisted extraction of antioxidative compounds from Bene (Pistacia atlantica subsp. mutica) hull using various solvents of different physicochemical properties, Food Chem. 173 (2015) 577–583, https://doi.org/10.1016/j.foodchem.2014.10.081.

[20] C.C. Liang, A.Y. Park, J.L. Guan, In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro, Nat. Protoc. 2 (2007) 329–333 doi:nprot.2007.30 [pii]\r10.1038/nprot.2007.30.

[21] E.B. Berens, J.M. Holy, A.T. Riegel, A. Wellstein, A Cancer cell spheroid assay to assess invasion in a 3D setting, J. Vis. Exp. (2015) e53409, https://doi.org/10.3791/53409.

[22] F. Busquet, R. Strecker, J.M. Rawlings, S.E. Belanger, T. Braunbeck, G.J. Carr, P. Cenijn, P. Fochtman, A. Gourmelon, N. Hübler, A. Kleensang, M. Knöbel, C. Kussatz, J. Legler, A. Lillicrap, F. Martínez-jerónimo, C. Polleichtner, H. Rzodeczko, E. Salinas, K.E. Schneider, S. Scholz, E. Van Den Brandhof, L.T.M. Van Der Ven, S. Walter-rohde, S. Weigt, H. Witters, M. Halder, OECD validation study to assess intra- and inter-laboratory reproducibility of the zebrafish embryo toxicity test for acute aquatic toxicity testing, Regul. Toxicol. Pharmacol. 69 (2014) 496–511, https://doi.org/10.1016/j.yrtph.2014.05.018.

[23] C. Yeh, Y. Liao, C. Chang, J. Tsai, Y. Wang, C. Cheng, C. Wen, Y. Chen, Caffeine treatment disturbs the angiogenesis of zebrafish embryos, Drug Chem. Toxicol. 35 (2012) 361–365, https://doi.org/10.3109/01480545.2011.627864.

[24] H.Z. Zhang, C.Y. Li, J.Q. Wu, R.X. Wang, P. Wei, M.H. Liu, M.F. He, Anti-angiogenic activity of para-coumaric acid methyl ester on HUVECs in vitro and zebrafish in vivo, Phytomedicine 48 (2018) 10–20, https://doi.org/10.1016/j.phymed.2018.04.056.

[25] N. Javadi, F. Abas, A.A. Hamid, S. Simoh, K. Shaari, I.S. Ismail, A. Mediani, A. Khatib, GC-MS-based metabolite profiling of Cosmos caudatus leaves possessing alpha-glucosidase inhibitory activity, J. Food Sci. 79 (2014) 1130–1136, https://doi.org/10.1111/1750-3841.12491.

[26] S. Bhatia, S.S. Tykodi, J.A. Thompson, Treatment of metastatic melanoma: an overview, Oncology 23 (2009) 488–496 http://www.ncbi.nlm.nih.gov/pubmed/19544689%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2737459.

[27] G.I. Evan, K.H. Vousden, Proliferation, cell cycle and apoptosis in cancer, Nature 411 (2001) 342–348, https://doi.org/10.1038/35077213.

[28] K. Vermeulen, D.R. Van Bockstaele, Z.N. Berneman, The cell cycle:a review of regulation,deregulation and therapeutic targets in cancer, Cell Prolif. 36 (2003) 131–149, https://doi.org/10.1306/74D715D2-2B21-11D7-8648000102C1865D.

[29] H. Ching, G.G. Chen, A.C. Vlantis, M.C.F. Tong, P.K.S. Chan, C.A. Van Hasselt, Induction of cell cycle arrest and apoptosis by 5-fluorouracil in laryngeal cancer cells containing HPV16 E6 and E7 oncoproteins, Clin. Biochem. 41 (2008) 1117–1125, https://doi.org/10.1016/j.clinbiochem.2008.06.007.

[30] C. Riccardi, I. Nicoletti, Analysis of apoptosis by propidium iodide staining and flow cytometry, Nat. Protoc. 1 (2006) 1458–1461, https://doi.org/10.1038/nprot.2006.238.

[31] S.-Y. Sun, N. Hail, R. Lotan, Apoptosis as a novel target for cancer chemoprevention, J. Natl. Cancer Inst. 96 (2004) 662–672, https://doi.org/10.1093/jnci/djh123.

[32] H. Lee, H. Cho, R. Yu, K. Lee, H. Chun, J. Park, Mechanisms underlying apoptosis-inducing effects of Kaempferol in HT-29 human colon cancer cells, Int. J. Mol. Sci. 15 (2014) 2722–2737, https://doi.org/10.3390/ijms15022722.

[33] M.O. Hengartner, The biochemistry of apoptosis, Nature 407 (2000) 770–776, https://doi.org/10.1038/35037710.

[34] S.L. Sankari, K.M.K. Masthan, N.A. Babu, T. Bhattacharjee, Mini-review apoptosis in cancer - an update, Asian Pac. J. Cancer Prev. 13 (2012) 4873–4878, https://doi.org/10.7314/APJCP.2012.13.10.4873.

[35] S.H. Kaufmann, W.C. Earnshaw, Induction of apoptosis by cancer chemotherapy, Exp. Cell Res. 256 (2000) 42–49, https://doi.org/10.1006/excr.2000.4838.

[36] D.B. Longley, D.P. Harkin, P.G. Johnston, 5-fluorouracil: mechanisms of action and clinical strategies, Nat. Cancer 3 (2003) 330–338, https://doi.org/10.1038/nrc1074.

[37] Y. Zhu, T. Ye, X. Yu, Q. Lei, F. Yang, Y. Xia, X. Song, L. Liu, H. Deng, T. Gao, C. Peng, W. Zuo, Y. Xiong, L. Zhang, N. Wang, L. Zhao, Y. Xie, L. Yu, Y. Wei, Nifuroxazide exerts potent anti-tumor and anti-metastasis activity in melanoma, Sci. Rep. 6 (2016) 20253, https://doi.org/10.1038/srep20253.

[38] A. Mali, U. Wagh, M. Hegde, S. Chandorkar, S. Surve, M. Patole, In vitro anti-metastatic activity of enterolactone, a mammalian lignan derived from flax lignan, and down-regulation of matrix metalloproteinases in MCF-7 and MDA MB 231 cell lines, Indian J. Cancer 49 (2012) 181–188, https://doi.org/10.4103/0019-509X.98948.

[39] A.H. Webb, B.T. Gao, Z.K. Goldsmith, A.S. Irvine, N. Saleh, R.P. Lee, J.B. Lendermon, R. Bheemreddy, Q. Zhang, R.C. Brennan, D. Johnson, J.J. Steinle, M.W. Wilson, V.M. Morales-Tirado, Inhibition of MMP-2 and MMP-9 decreases cellular migration, and angiogenesis in in vitro models of retinoblastoma, BMC Cancer 17 (2017) 1–11, https://doi.org/10.1186/s12885-017-3418-y.

[40] S. Valastyan, R. Weinberg, Tumor metastasis: molecular insights and evolving paradigms, Cell 147 (2011) 275–292, https://doi.org/10.1016/j.cell.2011.09.024.Tumor.

[41] C. Chuang, L. Wang, Y. Wong, E. Lin, Anti-metastatic effects of isolinderalactone via the inhibition of MMP-2 and up regulation of NM23-H1 expression in human lung cancer A549 cells, Oncol. Lett. (2018) 4690–4696, https://doi.org/10.3892/ol.2018.7862.

[42] I. Khan, P.S. Steeg, Metastasis suppressors: functional pathways, Lab Investig. 98 (2018) 198–210, https://doi.org/10.1038/labinvest.2017.104.

[43] J. Terriente, C. Pujades, Use of zebrafish embryos for small molecule screening related to cancer, Dev. Dyn. 242 (2013) 97–107, https://doi.org/10.1002/dvdy.23912.

[44] G.N. Serbedzija, E. Flynn, C.E. Willett, Zebrafish angiogenesis: a new model for drug screening, Angiogenesis 3 (2000) 353–359, https://doi.org/10.1023/A:1026598300052.

[45] M.M. Santoro, Antiangiogenic cancer drug using the zebrafish model, Arterioscler. Thromb. Vasc. Biol. 34 (2014) 1846–1853, https://doi.org/10.1161/ATVBAHA.114.303221.

[46] J. Zhang, B. Gao, W. Zhang, Z. Qian, Y. Xiang, Monitoring antiangiogenesis of bevacizumab in zebrafish, Drug Des. Devel. Ther. 12 (2018) 2423–2430, https://doi.org/10.2147/DDDT.S166330.

[47] Y. Mu, T. Yanase, Y. Nishi, A. Tanaka, Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells, Endocrinology 142 (2001) 3590–3597.

[48] E. Kim, J.H. Cho, E. Kim, Y.J. Kim, Ursodeoxycholic acid inhibits the proliferation of colon cancer cells by regulating oxidative stress and cancer stem-like cell growth,

PLoS One (2017) 1–11.
[49] T.C. Wan, F.Y. Cheng, Y.T. Liu, L.C. Lin, R. Sakata, Study on bioactive compounds of in vitro cultured Calculus suis and natural Calculus bovis, Anim. Sci. J. 80 (2009) 697–704, https://doi.org/10.1111/j.1740-0929.2009.00689.x.
[50] J. Li, C. Pan, T. Yanase, H. Nawata, Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell, Biochem. Biophys. Res. Commun. 303 (2003) 1002–1007, https://doi.org/10.1016/S0006-291X(03)00449-2.
[51] J. Collier, M. Frushour, K. Wilson, Cosmetic Composition and Methods of Use Thereof, 15 / 532, 411 (2018).
[52] A.A. Khan, A.M. Alanazi, M. Jabeen, A. Chauhan, A.S. Abdelhameed, Design, synthesis and in vitro anticancer evaluation of a stearic acid-based ester conjugate, Anticancer Res. 33 (2013) 2517–2524.
[53] L.M. Evans, S.L. Cowey, G.P. Siegal, R.W. Hardy, NIH public access, Nutr. Cancer 61 (2010) 746–753, https://doi.org/10.1080/01635580902825597.Stearate.
[54] S. Hardy, W. El-assaad, E. Przybytkowski, E. Joly, M. Prentki, Y. Langelier, Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells, J. Biol. Chem. 278 (2003) 31861–31870, https://doi.org/10.1074/jbc.M300190200.
[55] J. Reardon, T. Hussaini, M. Alsahafi, V.M. Azalgara, S.R. Erb, N. Partovi, E.M. Yoshida, Ursodeoxycholic acid in treatment of non-cholestatic liver diseases: a systematic review, J. Clin. Transl. Hepatol. 4 (2016) 192–205, https://doi.org/10.14218/JCTH.2016.00023.
[56] W. Huang, H. Hsu, J. Liu, T. Yang, C. Kuo, L. See, The association of ursodeoxycholic acid use with colorectal cancer risk, Med. (Baltimore) 95 (2016) 1–8, https://doi.org/10.1097/MD.0000000000002980.
[57] D. Lapenna, G. Ciofani, D. Festi, M. Neri, S.D. Pierdomenico, M.A. Giamberardino, F. Cuccurullo, Antioxidant properties of ursodeoxycholic acid, Biochem. Pharmacol. 64 (2002) 1661–1667.
[58] C.E. Marx, D.W. Bradford, R.M. Hamer, J.C. Naylor, T.B. Allen, J.A. Lieberman, J.L. Strauss, J.D. Kilts, Pregnenolone as a novel therapeutic candidate in schizophrenia: emerging preclinical and clinical evidence, Neuroscience 191 (2011) 78–90, https://doi.org/10.1016/j.neuroscience.2011.06.076.
[59] M.A. Elhinnawi, R.M. Mohareb, H.M. Rady, W.K.B. Khalil, M.M.A. Elhalim, G.A. Elmegeed, Novel pregnenolone derivatives modulate apoptosis via Bcl-2 family genes in hepatocellular carcinoma in vitro, J. Steroid Biochem. Mol. Biol. 183 (2018) 125–136, https://doi.org/10.1016/j.jsbmb.2018.06.006.
[60] A.S. Sivam, D. Sun-Waterhouse, S.Y. Quek, C.O. Perera, Properties of bread dough with added fiber polysaccharides and phenolic antioxidants: a review, J. Food Sci. 75 (2010), https://doi.org/10.1111/j.1750-3841.2010.01815.x.
[61] B. Huang, X. Ban, J. He, J. Tong, J. Tian, Y. Wang, Comparative analysis of essential oil components and antioxidant activity of extracts of Nelumbo nucifera from various areas of China, J. Agric. Food Chem. 58 (2010) 441–448, https://doi.org/10.1021/jf902643e.