CM & NH Cellular Medicine & Natural Health JOURNAL

Journal of Cellular Medicine and Natural Health
M. W. Roomi

A specific mixture of phytonutrients inhibits cell proliferation, secretion of MMPs and invasion through Matrigel in fibrosarcoma HT-1080 and melanoma A-2058 cells

M. W. Roomi, Ph.D., T. Kalinovsky, M.S., N. Jariwalla*, S. Siddiqui*, A. Niedzwiecki, Ph.D. and M. Rath, M.D.
Dr. Rath Research Institute, CA, USA

Abstract

Use of natural health products to prevent, inhibit and reverse carcinogenesis is gaining increasing importance, since scientific evidence shows that high consumption of fruits and vegetables are associated with a reduced risk of various cancers. PB, which is composed of quercetin, cruciferexTM, curcumin, green tea extract and resveratrol, was formulated by defining critical physiological targets in cancer progression and metastasis. We investigated the effect of PB on two different aggressive cancer cell lines- human fibrosarcoma cell line HT-1080 and melanoma A-2058- by evaluating cell viability, MMP secretion, invasion and morphology. Human fibrosarcoma HT-1080 and melanoma A-2058 cell lines were obtained from ATCC and cultured in DMEM media, supplemented with 10% FBS and antibiotics in 24 well tissue plates. At near confluence, cells were treated with PB at 0, 10, 25, 50, 75 and 100 µg/ml, in triplicate at each dose. Cells were also treated with phorbol 12-myristate 13-acetate (PMA) 100 ng/ml for MMP-9 induction. Cell proliferation was assayed by MTT assay, MMP by zymography, invasion through Matrigel and morphology by H&E staining. PB significantly inhibited proliferation of fibrosarcoma HT-1080 and melanoma A-2058 cells in a dose-dependent manner with 80% ($p=0.0001$) inhibition at 50-100 µg/ml concentration in HT-1080 cells and ~80% ($p<0.0001$) at 25-100 µg/ml in A-2058 cells. Zymography demonstrated MMP-2 and basal levels of MMP-9 in fibrosarcoma HT-1080 cells and strong induction of MMP-9 by PMA. MMP-2 and MMP-9 were inhibited by PB in a dose-dependent fashion with virtual blockage of both MMPs at 50 µg/ml. Zymography demonstrated MMP-2 in untreated melanoma A2058 cells and induction of MMP-9 by PMA. MMP-2 and MMP-9 were inhibited by PB in a dose-dependent fashion with virtual blockage of both MMPs at 50 µg/ml. PB completely blocked fibrosarcoma HT-1080 and melanoma A-2058 cell invasion through Matrigel at 25 µg/ml PB. H&E staining showed no morphological changes in HT-1080 or A-2058 cells exposed to PB at lower concentrations and slight changes at higher concentrations. These results suggest that PB is a potential therapeutic agent for fibrosarcoma and melanoma with potent antimetastatic activity, because it inhibited cell proliferation, MMP-2 and -9 secretion and invasion through Matrigel, important parameters for cancer prevention.

Keywords
phytonutrients, fibrosarcoma HT-1080, melanoma A-2058, cell growth, MMP-2 and-9, Matrigel invasion

Correspondence to
Dr. Aleksandra Niedzwiecki,
Dr. Rath Research Institute,
1260 Memorex Drive,
Santa Clara, CA 95050,
USA.

Email: author@jcmnh.org

*equally contributed

Introduction

Fibrosarcoma, an aggressive and highly metastatic cancer of connective tissue that primarily develops in metaphases of long tubular bones, is generally associated with poor prognosis (1,2). Cancer mortality usually results from tumor invasion of local tissue and metastasis to vital organs. About 3,300 new cases of bone cancer will be diagnosed and about 1,490 are expected to die from bone cancer in 2016 (1). About 4 % of bone cancers are fibrosarcomas (1,2). Skin cancer is the most common cancer. Though melanoma accounts for only about 1% of skin cancers, it is an extremely aggressive cancer, causing the most skin cancer-related deaths due to metastasis to other areas of the body, such as lymph nodes, lungs, liver, brain or bone (3). About 76,380 new melanomas will be diagnosed and 10,130 people are expected to die of melanoma in 2016 (3). The rates of melanoma have been rising for the last 30 years (3). Thus, any successful treatment for fibrosarcoma or melanoma must target metastasis. Degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) plays a critical role in the formation of tumors and metastasis and has been found to correlate with the aggressiveness of tumor growth and invasiveness of the cancer (4-6).

Polyphenols have been reported to provide a wide range of anticarcinogenic properties, such as modulating cell proliferation, tumor growth, angiogenesis, metastasis, inflammation and inducing apoptosis. Since polyphenols demonstrate poor bioavailability, combinations of several polyphenols have been proposed to modulate bioavailability of natural compounds and exercise pleiotropic effects. Thus, we investigated the anticarcinogenic effects of a specific mixture of polyphenols, including quercetin, CruciferexTM, curcumin, green tea extract and resveratrol on two different aggressive cancer cell lines in vitro: fibrosarcoma and melanoma.

Methods and Materials

Cancer cell lines and culture

Human fibrosarcoma HT-1080 and melanoma A-2058 cell lines were obtained from ATCC (American Type Culture Collection, Rockville, MD). HT-1080 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The media and sera used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL, Long Island, NY.

Composition of the phytonutrient mixture

The mixture of nutrients (PB) was composed of the following in the ratio indicated: quercetin 400 mg; cruciferex 400 mg; curcumin 300 mg; resveratrol 50 mg and standardized green tea extract (derived from green tea leaves),obtained from US Pharma Lab. The certificate of analysis indicated the following characteristics: total polyphenol 80%, catechins 60%, epigallocatechin gallate (EGCG) 35%, and caffeine 1.0%) 300 mg.

Cell Culture

Human fibrosarcoma HT-1080 cells and melanoma A-2058 were grown in DMEM, supplemented with 20% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in 24 well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 1 ml of media at 37º C in a tissue culture incubator equilibrated with 95% air and 5% CO_2. At near confluence, the cells were treated with PB, dissolved in media and tested at 0, 10, 25, 50, 75, and 100 µg/ml in triplicate at each dose. Phorbol 12-myristate 13-acetate (PMA) 100 ng/ml was added to cells to induce MMP-9 secretion. The plates were then returned to the incubator.

MTT Assay

Cell viability was evaluated by MTT assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. After 24h incubation, the cells were washed with phosphate buffered saline (PBS) and 500 µl of MTT (Sigma #M-2128), 0.5 mg/ml in media was added to each well. After MTT addition (0.5mg/ml) the plates were covered and returned to the 37º C incubator for 2h, the optimal time for formazan product formation. (Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 ml dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} of the control (treatment without supplement) was considered 100%.

Gelatinase Zymography

Gelatinase zymography was performed in 10% Novex Pre-Cast SDS Polyacrylamide Gel (Invitrogen Corporation) in the presence of 0.1% gelatin under non-reducing conditions. Culture media (20 µl) were mixed with sample buffer and loaded for SDS-PAGE with tris glycine SDS buffer, as suggested by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 minutes at room temperature to remove the SDS. The gels were then incubated at 37º C overnight in a substrate buffer containing 50mM Tris-HCl and 10mM $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes and destained. Upon renaturation of the enzyme, the gelatinases digested the gelatin in the gel, producing clear bands against an intensely stained background. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Matrigel Invasion

Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24 well plates. Suspended in medium, HT-1080 or A-2058 cells were supplemented with nutrients, as specified in the design of the experiment and seeded on the insert in the well, thus both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 hours. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin and visually counted under the microscope.

Morphology: H&E staining

Morphology of cells cultured for 24h in test concentrations of NM were evaluated by H&E staining and observed and photographed by microscopy.

Statistical Analysis

The results were expressed as means + SD, as indicated in the results, for the groups. Data was analyzed by independent sample "t" test using MedCalc Software (Markakerke, Belgium).

Results

Cytotoxicity: MTT assay 24h

PB significantly inhibited proliferation of fibrosarcoma HT-1080 cells in a dose-dependent manner with 50% (p=0.001) at 10 µg/ml, 60% (p=0.0005) at 25 µg/ml and 80% (p=0.0001) at 50-100 µg/ml concentration, linear trend R^2 = 0.7391. Similarly, PB showed potent significant inhibition of melanoma A-2058 cells with increasing concentration, demonstrating 45% (p=0.0001) inhibition at 10 µg/ml and ~80% (p<0.0001) at 25-100 µg/ml concentration, linear trend, R^2 = 0.6981. See Figures 1A and 1B.

Gelatinase zymography

Zymography demonstrated MMP-2 and basal levels of MMP-9 in fibrosarcoma HT-1080 cells and strong induction of MMP-9 by PMA. MMP-2 and MMP-9 were inhibited by PB in a dose-dependent fashion with virtual blockage of both MMPs at 50 µg/ml, as shown in Figure 2. Zymography demonstrated MMP-2 in untreated melanoma A2058 cells and induction of MMP-9 by PMA. MMP-2 and MMP-9 were inhibited by PB in a dose-dependent fashion with virtual blockage of both MMPs at 50 µg/ml, as shown in Figure 3.

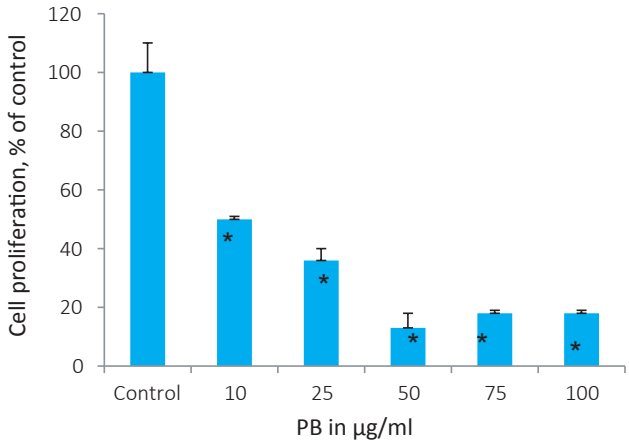

Figure 1A – Effect of PB on growth of fibrosarcoma HT-1080: MTT assay 24h

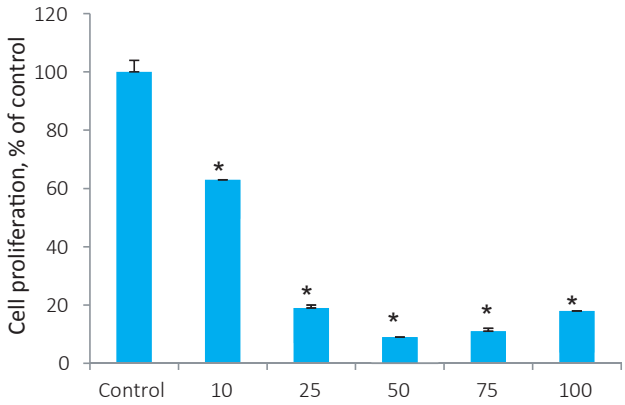

Figure 1B – Effect of PB on growth of melanoma A-2058: MTT assay 24h

2A – Untreated HT-1080 cells

2B –PMA (100 ng/ml)-treated HT-1080 cells

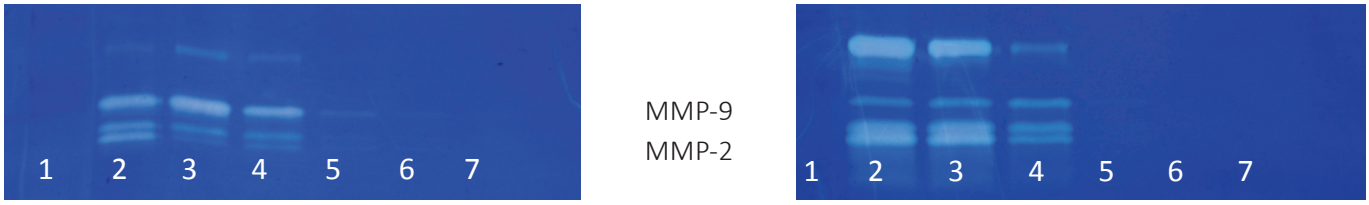

Figure 2 – Effect of PB on MMP-2 and -9 in normal (2A) and PMA (100ng/ml) –treated (2B) fibrosarcoma HT-1080 cells: gelatinase zymography
Legend: 1 -Markers, 2- Control, 3-7 PB 10, 25, 50, 75, 100 µg/ml

3A – Untreated A-2058 cells

3B –PMA (100 ng/ml)-treated A-2058 cells

Figure 3 – Effect of PB on MMP-2 and -9 in normal (3A) and PMA (100ng/ml) –treated (3B) melanoma A-2058 cells: gelatinase zymography
Legend: 1 -Markers, 2- Control, 3-7 PB 10, 25, 50, 75, 100 µg/ml

Matrigel invasion

PB inhibited fibrosarcoma HT-1080 cell invasion through Matrigel by 100% at 25 µg/ml, as shown in Figure 4. Melanoma A-2058 Invasion through Matrigel was inhibited by 65% at 10 and 100% at 25 µg/ml PB, as shown in Figure 5.

Morphology: H&E staining

H&E staining showed no morphological changes in fibrosarcoma HT-1080 nor in melanoma A-2058 cells exposed to PB at lower concentrations and slight changes at higher concentrations, as shown in Figures 6 and 7.

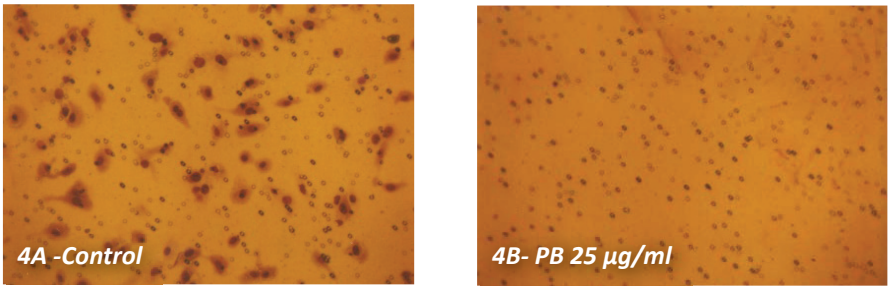

Figure 4 – Effect of PB on Matrigel invasion of fibrosarcoma HT-1080 cells

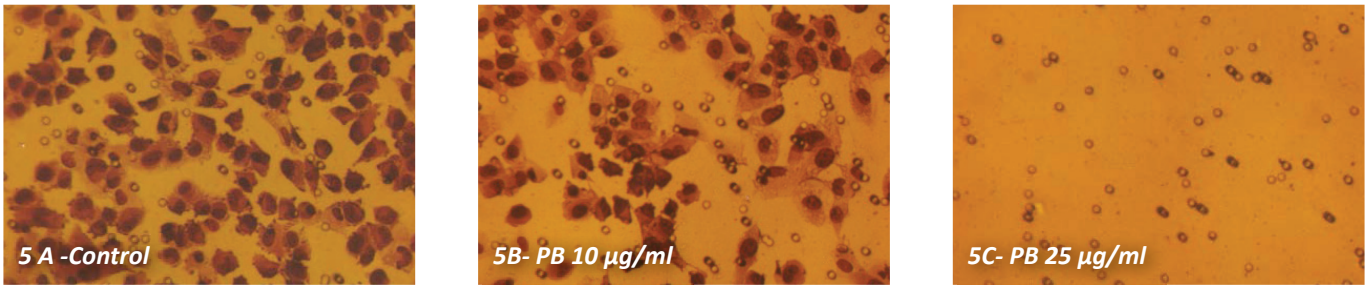

Figure 5 - Effect of PB on Matrigel invasion of melanoma A-2058 cells

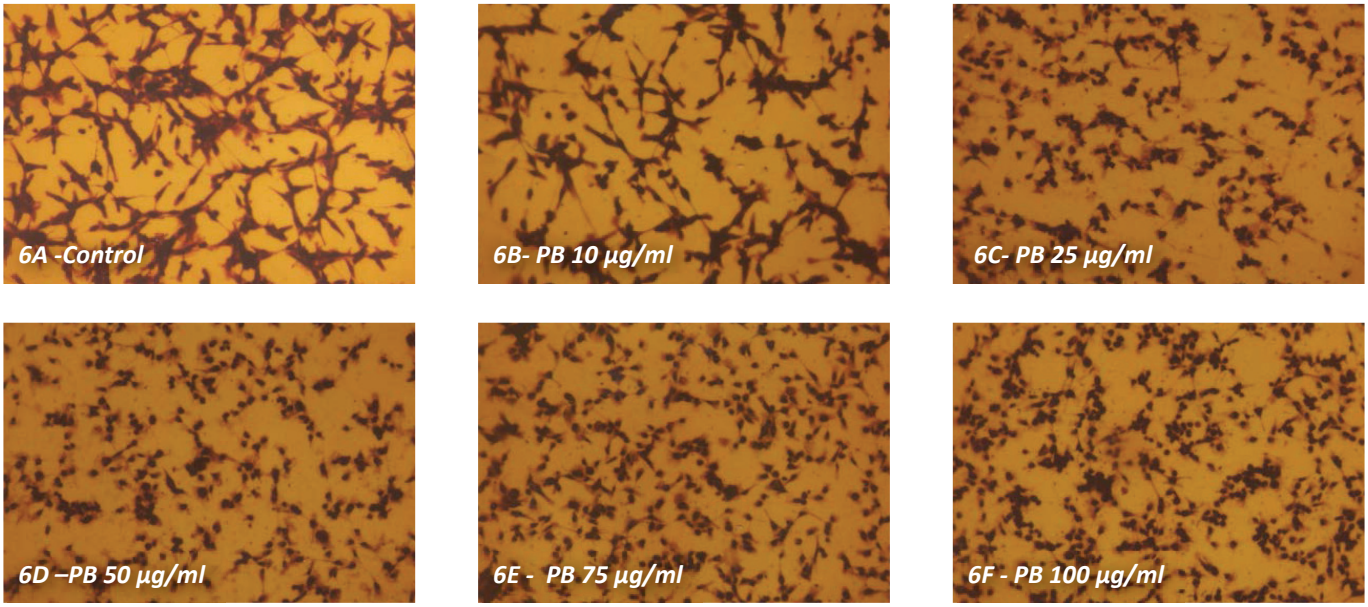

Figure 6 – Effect of PB on morphology of fibrosarcoma HT-1080 cells: H&E staining

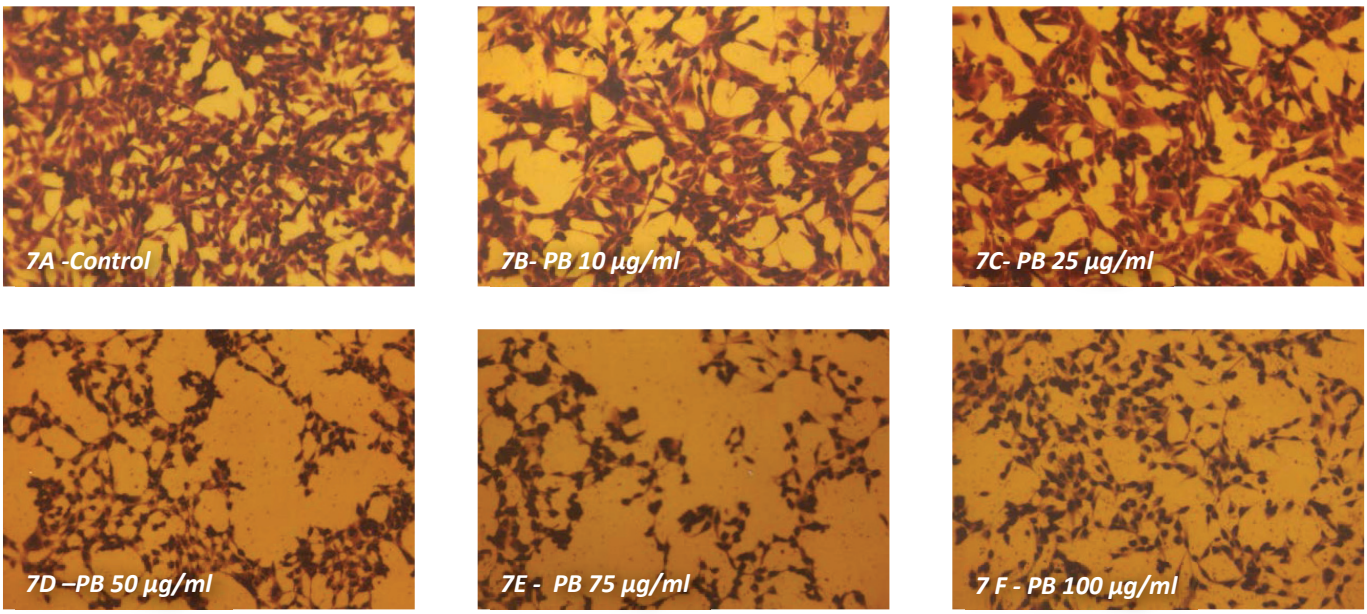

Figure 7 – Effect of PB on morphology of melanoma A-2058 cells: H&E staining

Discussion

The results from our *in vitro* study show that the combination of polyphenols in PB has significant anti-cancer effects against both fibrosarcoma HT-1080 and melanoma A-2059 cells. As such, PB demonstrated inhibitory effects on key parameters involved in cancer cells' invasion and spread. The secretion of key ECM proteinases associated with cancer invasion and metastasis MMP-2 and MMP-9 was completely blocked by PB at 50 µg/ml in both cell lines and invasion of cells through Matrigel was 100% inhibited at 25 µg/ml. In addition, PB demonstrated increased cellular toxicity in both cell lines in a dose-dependent manner, with 80% inhibition of HT-1080 cells and A-2058 cells at 50-100 µg/ml and at 25-100 µg/ml, respectively.

Scientific evidence demonstrates a positive correlation between high consumption of fruits and vegetables and reduced risk of various cancers, leading to the increasing focus on using natural health products to prevent, inhibit and reverse carcinogenesis (7,8). The phytonutrient mixture used in this study was formulated with the aim of achieving wide pleiotropic effects against cancer by simultaneously targeting critical cellular mechanisms involved in cancer progression and metastasis. Curcumin has been reported to affect multiple mechanisms involved in various types of cancers, such as leukemia, lymphoma, gastrointestinal cancers, genitourinary cancers, breast cancer, ovarian cancer, head and neck squamous cell carcinoma, lung cancer, melanoma, neurological cancers and sarcomas (9). It has been shown to act by inhibiting cancer cell proliferation, invasion (MMP-9 and adhesion molecules), angiogenesis and metastasis, and inducing apoptosis (9). Cruciferex™, containing extracts from cruciferous vegetables (cabbage, cauliflower, and broccoli) and carrots, displays wide anti-carcinogenic activity such as detoxification/excretion of carcinogens, protection against oxidative stress and inhibition of cancer cell proliferation and induction of apoptosis (10). Green tea extract has been shown to modulate cancer cell growth, metastasis, angiogenesis and other cellular mechanisms involved in cancer progression (11-15). Another PB constituent, resveratrol, has been shown to inhibit cancer cell proliferation, modulate signal transduction pathways that control

cell division and growth, induce apoptosis and modulate inflammation, angiogenesis and metastasis (16,17). Cancer preventive effects of quercetin include induction of cell cycle arrest, apoptosis and antioxidant functions (18). Combinations of quercetin (from onion) and green tea positively affected the bioavailability of green tea catechin, EGCG. (19)

Conclusion

Current treatment methods for aggressive metastatic cancers such as fibrosarcoma and melanoma are generally ineffective and toxic. Thus, a need exists for developing effective and non-toxic therapeutic agents for these cancers. Our studies demonstrated that the mixture of the non-toxic polyphenolic compounds in PB significantly inhibited cancer-invasive parameters in fibrosarcoma HT-1080 and melanoma A-2058 cell lines, such as secretion of MMP-2 and-9, cell invasion, as well as cell proliferation. These results show that PB has a potential to become a natural, non-toxic therapeutic agent for fibrosarcoma and melanoma.

Acknowledgments

The research study was funded by Dr. Rath Health Foundation (Santa Clara, CA), a non-profit organization.

References

1. American Cancer Society: Bone cancer. What are the key statistics about bone cancer? http://www.cancer.org/cancer/bonecancer/detailedguide/bone-cancer-key-statistics. Accessed 10/13/16, Last revised 1/21/16.

2. Papagelopoulos PJ, Galanis E, Frassica FJ, Sim FH, Larson DR, Wold LE. Primary fibrosarcoma of bone. Outcome after primary surgical treatment. Clin Orthop Relat Res. 2000; 373: 88-103.

3. American Cancer Society: Skin cancer. Key statistics for melanoma/skin cancer. http://www.cancer.org/cancer/skincancer-melanoma/detailedguide/melanoma-skin-cancer-key-statistics Last revised: 5/20/2016; accessed 10/13/16

4. Fidler IJ. Molecular biology of cancer: invasion and metastasis. In: De Vita VT, Hellman S, Rosenberg SA (eds). Cancer Principles and Practice of Oncology. 5th ed. Philadelphia, PA: Lippincott-Raven, 1997: 135-152.

5. Duffy MJ. The role of proteolytic enzymes in cancer invasion and metastasis. Clin Exp Metastasis. 1992; 10: 145-155.

6. Stetler-Stevenson WG. The role of matrix metalloproteinases in tumor invasion, metastasis and angiogenesis. Surg Oncol Clin N Am. 2001; 10, 383-392.

7. Aldercreutz H: Western diet and Western disease: some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest Suppl. 1990; 201: 3-23.

8. Miller AB. Diet and Cancer: A review. Acta Oncologica. 1990; 29(1): 87-95.

9. Anand P, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB. Curcumin and cancer: An "old-age" disease with an "age-old solution. Cancer Letters. 2008; 267:133-164.

10. Keck AS, Finley JW: Cruciferous vegetables: cancer protective mechanisms of glucosinolate hydrolysis products and selenium. Integrative Cancer Therapies. 2004; 3(1): 5-12.

11. Valcic S, Timmermann BN, Alberts DS, et al. Inhibitory effect of six green tea catechins and caffeine on the growth of four selected human tumor cell lines. Anticancer Drugs. 1996; 7:461-8.

12. Mukhtar H, Ahmed N. Tea polyphenols: prevention of cancer and optimizing health. Am J Clin Nutr 2000; 71:1698-1702.

13. Yang GY, Liao J, Kim K, Yurtow EJ, Yang CS. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. Carcinogenesis. 1998; 19: 611-616.

14. Taniguchi S, Fujiki H, Kobayashi H, et al. Effect of (-) epigallocatechin gallate, the main constituent of green tea, on lung metastasis with mouse B16 melanoma cell lines. Cancer Lett. 1992; 65: 51-54.

15. Harakeh S, Abu-El-Ardat K, Diab-Assaf M, Niedzwiecki A, El-Sabban M, Rath M. Epigallocatechin-3-gallate induces apoptosis and cell cycle arrest in HTLV-1-positive and-negative leukemia cells. Med Oncol. 2008; 25(1):30-39.

16. Cal C, Garban H, Jazirehi A, Yeh C, Mizutani Y, Bonavida B. Resveratrol and cancer chemoprevention, apoptosis and chemo-immunosensitizing activities. Curr Med Chem - Anti-Cancer Agents. 2003; 3: 77-93.

17. Bishayee A. Cancer prevention and treatment with resveratrol: from rodent studies to clinical trials. Cancer Prev Res. 2009; 2(5): 409-417.

18. Gibellini L, Pinti M, Nasi M, et al. Quercetin and cancer chemoprevention. Evidence-Base Complementary and Alternative Medicine. 2011: Article ID# 591356.

19. Kale A, Gawande S, Kotwal S, et al. Studies on the effects of oral administration of nutrient mixture, quercetin and red onions on the bioavailability of epigallocatechin gallate from green tea extract. Phytotherapy Research. 2010; 24(S1); 48-55.