Published in final edited form as:

Nutr Cancer. 2014 ; 66(6): 930–937. doi:10.1080/01635581.2014.922201.

The effect of black raspberry extracts on MnSOD activity in protection against concanavalin A induced liver injury

Xuanyi Li¹, Yan Li¹, Vanessa A. States¹, Suping Li¹, Xiang Zhang², and Robert C.G. Martin¹

¹Division of Surgical Oncology, Department of Surgery, University of Louisville, Louisville, KY 40202

²Department of Chemistry, University of Louisville, Louisville, KY 40202

Abstract

Inflammation and oxidative stress are the key events in carcinogenetic transformation. Black raspberries (BRB) have been demonstrated to have antioxidant, anti-inflammatory and anti-cancer bioactivities. In this study, a concanavalin A induced hepatitis mouse model is used to examine the effect of BRB extract on hepatic injury. Three BRB extracts, including ethanol/H₂O extracts (both anthocyanin-contained fraction and non-anthocyanin-contained fraction) and hexane extract were used. The alterations in hepatic histology, apoptosis and oxidative stress were observed in the animals pretreated with BRB extracts and then challenged by concanavalin A. Results indicate that ethanol/H₂O extracts can inhibit Con A induced liver injury. The hepatic protection by the ethanol/H₂O BRB extracts is associated with decreases of lipid peroxidation and NDA oxidative damage. Importantly, the BRB extracts increase manganese superoxide dismutase (MnSOD) activity but not the CuZnSOD. The preservation of MnSOD by BRB extracts is associated with the protective action in the liver challenged by Con A. Ethanol/H₂O BRB extracts function as antioxidants, thus demonstrating the critical role of oxidative stress in the Con A induced liver injury, and providing evidence that the protective effects of ethanol/H₂O BRB extracts result, at least in part, from their antioxidant action.

Keywords

Black raspberries; Superoxide Dismutase; Hepatitis

Introduction

Hepatocellular carcinoma (HCC) is currently the fifth most common form of cancer and the third leading cause of cancer death worldwide ¹. As 80–90% of HCC patients suffer from prior liver conditions, chronic hepatitis, cirrhosis, hepatitis B virus (HBV) and hepatitis C virus (HCV) are major risks factors for the development of HCC ². An amplified level of oxidative stress is a suggested cause of liver damage in chronic viral hepatitis (B and C) ^{3,4}, hepatitis in occupational exposure to toxicity⁵, alcoholic liver disease⁶, and non-alcoholic

Address for Correspondence: Robert C.G. Martin, MD, PhD, Department of Surgery, Division of Surgical Oncology, University of Louisville School of Medicine, 315 E. Broadway - #312, Louisville, KY 40202, Ph 502-629-3355, Fax 502-629-3030, robert.martin@louisville.edu.

steatohepatitis⁷. Chronic inflammation and persistent hepatic oxidative damage (i.e., oxidative DNA damage) are frequently associated with HCC development⁸. Evidence also indicates that the antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione, are significantly lower patients with chronic hepatitis^{9;10}.

Despite encouraging reports regarding antiviral therapy for infection with HBV or HCV and treatment of HCC, the effectiveness in clinical practice is low¹. It is tremendously important to explore a novel strategy for HCC prevention and therapy. On the basis of the epidemiological studies, natural products, among their many biological and pharmacological properties, have been suggested to possess anticancer potential¹¹. Natural products could be beneficial supplements to current cancer therapies because they can potentially increase the efficacy of treatments while causing less harm to the patient. However, there is a significant gap in the current knowledge of how the natural products protect against chronic liver injury and prevent the carcinogenetic transformation of HCC.

The consumption of black raspberries (BRB), a natural food product with antioxidant and anti-inflammatory bioactivities, is associated with lower incidences of esophageal and colon cancer in animal studies¹². Previous studies indicate that BRB extracts reduce survival of human breast cancer cells by inhibiting NFκB dependent radiation resistance¹³. BRB can also inhibit intestinal tumorigenesis in Apc1638+/- and Muc2-/- mouse models of colorectal cancer¹⁴. Another study identified cyaniding glycosides as constituents of freeze-dried black raspberries which inhibit anti-benzo[a]pyrene-7,8-diol-9,10-epoxide induced NFκB and AP-1 activity¹⁵. Blueberries, strawberries, and black raspberries all contain anthocyanins, which give them their characteristic colors and may contain chemopreventive properties¹⁶. In fact, the anticancer potential of these berries is related to a multitude of bioactive phytochemicals, including polyphenols (flavonoids, proanthocyanidins, ellagitannins, gallotannins, phenolic acids), stilbenoids, lignans, and triterpenoids. Studies show that the anticancer effects of berry bioactives are partially mediated through their abilities to counteract, reduce, and also repair damage resulting from oxidation¹⁷. With the properties of anti-oxidative stress and anti-inflammation, these colorful fruits could be important resources for finding a novel phytochemical-based strategy for hepatic protection. However, the effects of these berries on the prevention of liver damage have not been studied.

In this study, we proposed to use a mouse model to examine the effect of BRB extract on hepatitis. Concanavalin A (Con A) a lectin with mannose specificity can induce acute hepatic inflammation, as a model of hepatitis¹⁸. The alterations in histology, apoptosis, oxidative damages, and antioxidants were observed. The aim of this study is to record the cellular and molecular events associated with Con A induced hepatitis in mice by the administration of the BRB extracts supplementation.

Materials and Methods

Male 25–30 g C57BL/6J mice (Jackson labs, Bar Harbor, ME) were housed three per cage, given commercial chow and tap water, and maintained on a 12-hour light/dark cycle. They were allowed to acclimate for 2 weeks prior to treatments. Three BRB extracts were gifted

from Dr. Gary D. Stoner (Ohio State University), including BRB-A (ethanol/H₂O extraction-soluble fraction), BRB-B (hexane extraction), and BRB-C (ethanol/H₂O extraction-insoluble extract). The animals were divided into 5 groups. The BRB pretreated groups were gavaged with BRB-A, BRB-B, and BRB-C at 1.6g/kg body weight/day (40 mg/25 g mouse) for 3 days. The dosage is equivalent to the diet containing freeze-dried BRB at 10% BRB, which has shown a therapeutic or preventive effect on the pathogenesis of ulcerative colitis and related neoplastic events¹⁹. Three days after BRBs treatment, the animals were treated with Con A (i.v.) at 30 mg/kg. The Con A hepatic injury group was pretreated (gavage) with saline in the same amount as BRB pretreated groups for 3 days and then treated with Con A (i.v.) at 30 mg/kg. The normal control group was pretreated with saline gavage for BRBs for 3 days and then treated with saline (i.v.) same amount as the Con A. Twelve hours after the administration of Con A, the mice were euthanized by over dose of anesthesia.

The serum was collected for the measurement of alanine aminotransferase, and the liver tissue was harvested for the measurements of histology, apoptosis, lipid peroxidation, 8-hydroxy-deoxyguanosine (8-OH-dG), and the antioxidants enzymatic activities, including SODs, glutathione and catalase. This study was approved by the Institutional Animal Care and Use Committee at the University of Louisville. Alanine aminotransferase (ALT) enzymatic activity of serum samples was determined using an ALT (GPT) Reagent kit according to the instruction provided.

Samples of liver tissue from each mouse were fixed in 10% buffered formalin for 48 hours and transferred into 80% ethanol. The formalin fixed tissue was embedded in paraffin. Five-µm sections were mounted onto glass slides and stained with hematoxylin and eosin for histopathologic analysis. Histopathology changes including pericentral and lobular inflammation and centrilobular necrosis, which were graded on a severity scale from 0 to 3 (0 = no lesion, 1 = mild, 2 = moderate, 3 = severe).

TdT Labeling (TUNEL) assay

ApopTag® in-situ apoptosis detection kit (EMD Millipore, MA) was used to detect the apoptotic cells according to the product instruction. Five-mm thick sections were cut from the paraffin blocks. After deparaffinization and rehydration, endogenous peroxidase was blocked with H₂O₂ in methanol for 20 minutes. The sections underwent proteinase K digestion for 15 minutes. DNA fragments were tailed using digoxigenin-dUTP along with anti-digoxigenin antibody conjugation with horseradish peroxidase (HRP) along with the substrate (DAB-H₂O₂) to develop a brown color. An apoptotic index (number of TUNEL positive epithelial nuclei/number of total epithelial nuclei) was calculated.

Immunohistochemical assays

Immunohistochemical staining was carried out on the paraffin-embedded material using the DAKO EnVision+System Kit. In brief, the sections were deparaffinized and hydrated. The slides were washed with a TRIS-buffer, and peroxidase blocking was performed for 5 minutes. After rewashing, the polyclonal rabbit Ab-8-OH-dG was applied for 60 minutes and then incubated with labeled polymer for 30 minutes at room temperature. The substrate-

chromogen solution (diaminobenzidine) was added as a visualization reagent. The digital images of 8-OH-dG staining was acquired with the microscope at 40× magnification using the Spot camera via the MetaMorph® Imaging System (Universal Imaging Corporation, Downingtown, PA) and stored as JPG data files (the resolutions were fixed as 200 pixels/inch). The procedure for the computer image analysis was performed, and the acquired color images from the immunohistochemical staining were defined a standard threshold according to the software specification. The computer program then quantified the threshold area represented by color images. 8-OH-dG levels are defined by the percentages of threshold area in acquired color images.

Lipid peroxidation assay

Lipid peroxidation is quantified by an OXItek TBARS Assay kit (ZeptoMetrix Corp.) measuring the malondialdehyde (MDA) concentrations as described in the provided instruction (Thiobarbituric acid–reactive substance assay). Briefly, the tissue homogenate is processed for thiobarbituric acid reaction following the procedure described. The reaction mixture is covered and incubated at 95°C for 60 minutes and then cooled to room temperature in an ice bath for 10 minutes. The samples were centrifuged at 3,000 rpm for 15 minutes, and the absorbance is obtained with a microplate reader reading at 532 nm.

Assay for enzymatic activities of SODs

SOD enzymatic activity was determined by a SOD assay kit-WST (Dojindo Molecular Technologies, INC.) as previously described.^{16, 29} Briefly, a colorimetric assay is done by measuring the a water soluble formazan dye, which produced by the reaction between WST-1 and superoxide anion (O_2^-). The rate of the reduction with O_2^- was linearly related to the xanthine oxidase activity and is inhibited by SOD. The absorbance was obtained with a microplate reader reading at 450 nm. MnSOD activity is determined by adding 1 mmol/L potassium cyanide (KCN) to samples to block the Cu/ZnSOD activity completely and then subtracting the Cu/ZnSOD activity from the total SOD activity.

Assays for Glutathione and Catalase

Glutathione is determined by a Cayman's GSH assay kit (Cayman Chemical, Ann Arbor, MI) utilizing an enzymatic recycling method. In brief, using the sulfhydryl group of GSH reacts with DTNB (5,5'-dithio-bis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which in turn is directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 412 nm provides an accurate estimation of GSH in the sample. Because of the use of glutathione reductase in this assay, both GSH and GSSG are measured and the assay reflects total glutathione. Catalase was determined by a Cayman's GSH assay kit (Cayman Chemical, Ann Arbor, MI) utilizing the peroxidatic function of catalase for determination of enzyme activity. In brief, the method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2 . The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the

chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color. Measurement of the absorbance at 540 nm provides an accurate estimation of catalase activities in the sample.

In Vitro studies

Hepatocytes were isolated from male SD rats by a collagenase (type-4) perfusion method. In brief, the collagenase-perfused liver was dissected, and suspended in Hanks solution, and filtered through 100- μ m nylon mesh. The purified hepatocytes were seeded in the polystyrene plates (96-well and 6-well) to study the effect of BRB extracts on the hepatocytes. It has been demonstrated that hypoxia/reoxygenation-caused necrotic cell death is associated with mitochondrial oxidative stress in hepatocytes²⁰; hepatocyte hypoxia/reoxygenation was performed to induce mitochondrial oxidative stress. Hepatocytes were pretreated by with BRB extracts at 100 μ g/ml along with a MnSOD mimic manganese (III) tetrakis(4-benzoic acid) porphyrin (MnTBAP) at 50 μ M for 12 hours. To simulate ischemia, hepatocytes were incubated in the medium at pH 6.2 in an anaerobic chamber for 4 hours. To simulate the reoxygenation and return to physiological pH of reperfusion, the anaerobic medium at pH 6.2 was replaced with aerobic medium at pH 7.4. Four hours after hypoxia/reoxygenation, the cell viability, MnSOD activity, and MnSOD protein level were determined by MTT assay, enzymatic assay and Western blot analysis respectively.

Statistical analysis

SPSS version 16.0 was used for data analysis. Continuous variables were compared using a t-test. Values were expressed as mean $\pm$ standard deviation. A two tailed p-value <0.05 was considered significant.

Results

BRB extracts protect against Con A induced liver damage

Con A administration resulted in pronounced damages in the hepatic tissues. Histological analysis by H&E staining revealed that damage with a multifocal distribution of hepatic cell death and inflammatory infiltration in large areas of the liver. The severity of hepatic damage is consistent with the increased levels of serum ALT activity. Treatment with BRB-A and BRB-C showed attenuation of the Con A induced hepatic injury, but not the BRB-B group. There was still extensive distribution of hepatic cell death and inflammatory infiltration in the BRB-B pretreated group. Consistently, BRB-A and BRB-C attenuated the increased levels of serum ALT activity by Con A administration, but not the BRB-B pretreated group. The representative histological changes and analysis were shown in figure 1A. A histological scoring was carried out on the H&E staining slide to evaluate the degree of hepatitis. After cells were treated with Con A, the histological score of inflammatory infiltration was 2.3 ± 0.82 , while the cell death was 2.67 ± 0.52 . The histopathology scores for the groups of Con A+A, Con A+B and Con A+C are 1.2 ± 0.84 , 2.5 ± 0.84 , and 1.16 ± 0.75 respectively. The cell death scores of three groups (Con A+A, BRB-B and BRB-C) are 1.2 ± 0.45 , 2.67 ± 0.51 and 1.33 ± 0.51 (figure 1B). Likewise, Con A treated mice showed markedly increased serum ALT value at 206.52 ± 65.45 U/L. The ALT levels in the BRB-A and BRB-C groups were significantly attenuated to 65.23 ± 32.4 U/L and 70.56

± 33.5 , but there was no statistically significant decrease in the BRB-B group ($p > 0.05$ compared to the Con A group) even though it was down to 177.96 ± 45.61 U/L. The ALT level is shown in figure 1C.

Prevention of cell death by BRB extracts associated with the decreased oxidative damage

The apoptotic index derived by counting positive TUNEL staining hepatocytes in Con A challenged liver tissue significantly increased in comparison to the controls. In the BRB-A and BRB-C treated groups, however, the apoptotic index was significantly attenuated compared to that in the Con A mice. The apoptotic cells represented by the positive TUNEL staining were shown in figure 2A. Numerous apoptotic cells were found in the Con A challenged liver tissues; however, treatment with BRB-A and BRB-C significantly reduced the numbers of apoptotic cells. The apoptotic index was shown in figure 2B. Two measurements regarding oxidative stress were performed in the liver tissues: TBARS for lipid peroxidation and 8-hydroxy-deoxyguanosine (8-OH-dG) for oxidative damage to DNA. The results of TBARS and 8-OH-dG indicated that MDA level and 8-OH-dG level were considerably increased in liver tissues by Con A treatment. Treatment with BRB-A and BRB-C significantly reduced the levels of lipid peroxidation products and DNA damages in liver tissues; however, BRB-A treatment did not affect the lipid peroxidation and 8-OH-dG. The results of TBARS and 8-OH-dG showed in figures 2C and 2D.

Preservation of MnSOD activity by BRB extracts treatment

The cellular antioxidant enzymes, including MnSOD, CuZnSOD, GSH and catalase were measured by enzymatic activity analysis. For the SODs, the total activity was significantly decreased in the Con A group, but it was similar among all other groups. MnSOD activity was significantly inhibited in all the samples of Con A treated groups. Treatment with BRB-A and BRB-C attenuated MnSOD inhibition by Con A. Unlike MnSOD, CuZnSOD activity showed a reverse trend compared to MnSOD, whereas the increased activity was found in the Con A group. The CuZnSOD showed less activity in the groups of BRB-A and BRB-C. The protein level of GSH was lowered in the Con A group, and treatment with the BRB extracts did not augment the levels of GSH ($P > 0.05$). The catalase enzymatic activities were also measured in the liver tissues; however, no significant difference was found among the study groups ($P > 0.05$). The cellular antioxidant enzymes are shown in figure 3.

MnSOD could be responsible for the protection of hepatocytes by BRB extracts

To confirm the *in vivo* data that MnSOD could be responsible for the hepatocytic protection, we tested the protective effect of BRB extracts in cultured hepatocytes. After ischemia/reoxygenation, necrotic cell killing increased to more than 50% in hepatocytes within 4 hours. Hepatocytes were protected by the pretreatment of BRB-A and BRB-C. Similar to the BRB extracts, MnTBAP was also showed protective action when the hepatocytes were challenged by ischemia/reoxygenation (figure 4 A). Both MnSOD protein level and enzymatic activity were preserved by treatments of BRB-A and BRB-C in the hepatocytes challenged by ischemia/reoxygenation (figure 4 B).

Discussion and Conclusion

Inflammation and oxidative damage have been accepted as driving forces for carcinogenetic transformation²¹. Our results have shown that ethanol/H₂O BRB extracts are endowed with antioxidant properties that are protective in the liver against the deleterious insults of Con A. Cell death in hepatic injury via Con A is associated with increased oxidative damage and decreased cellular antioxidants in the liver. There is a significant loss of MnSOD activities, which contribute to the loss of total SOD activity in hepatic tissues by Con A challenge. Ethanol/H₂O BRB extracts inhibit Con A induced liver injury, which is associated with preservation of MnSOD and decreases of lipid peroxidation and NDA oxidative damage. Thus, this study demonstrates the critical role of oxidative stress in Con A induced liver injury, and provides evidence that the protective effect of BRB extracts is, at least in part, from their antioxidant action.

Our results are in agreement with previous findings that, 1) BRB diet can significantly reduce hormone 17 β -estradiol E(2)-induced high levels of 8-oh-dG in the liver²²; and 2) black berry juice can protect the liver from fluorosis insults through increased antioxidants, including SOD, catalase, total antioxidant capacity and glutathione. Importantly, we find that MnSOD is preserved by pretreatment of BRB in the compromised liver when challenged by Con A. As we know, MnSOD constitutes a major protective mechanism against ROS by removing O₂[•] from both mitochondrial and the cytosol. Active MnSOD is located in the mitochondria, primarily concerned with intracellular energy homeostasis. In fact, mitochondria also play crucial roles in regulation of apoptotic and necrotic cell death.

In current study, we used a Con A-liver injury model, which has been demonstrated as a cellular event of T-cell activation²³. The liver injury is associated with the release of a variety of cytokines, including interleukin 1 β (IL-1 β), IL-2, IL-4, IL-5, interferon gamma (IFN- γ), and tumor necrosis factor alpha (TNF- α)^{24–26}. By antibody treatment, TNF- α and IFN- γ have been proven to play critical roles in the hepatocyte death^{25;26}.

The hepatocyte protection by BRB extracts preserving MnSOD in Con A challenged liver is supported by the following observations, 1) an increase in the intracellular steady-state production of H₂O₂ by MnSOD can protect against tumor necrosis factor induced apoptosis²⁷; 2) overexpression of MnSOD inhibited IFN- γ -mediated ROS accumulation and rescued cells from necroptosis²⁸; 3) in our previous study, we have found that supplementation with a MnSOD mimic-MnTBAP-can also protect the hepatocytes from Con A induced hepatitis²⁹; 4) in the current *in vitro* data, supplementation with a MnSOD mimic-MnTBAP-can also protect the hepatocytes, while abolishing the MnSOD activity by KCN cannot protect the liver of animals pretreated with BRB.

In this study, we used three BRB extracts, including BRB-A (ethanol/H₂O-soluble fraction), BRB-B (hexane extract, mainly nonpolar compounds), and BRB-C (ethanol/H₂O-insoluble fraction). The ethanol/H₂O-soluble fraction is the anthocyanin-contained fraction, while ethanol/H₂O-insoluble fraction is the non- anthocyanin-contained fraction, according to the Stoner group report³⁰. Our data show that both the soluble and insoluble fractions by ethanol/H₂O extraction can protect the liver from Con A insult, but not the hexane extract of

BRB. Our finding is again consistent with that of the Stoner group³⁰. This result demonstrates that multiple compounds in BRB could be potential as the phyto-chemicals based therapeutic agents to protect the liver injury. In spite of the bio-active properties, most natural products have not yet been approved as therapeutic agents, and the low bioavailability has been highlighted as a major problem for this. For example, in our previous studies, we used 2.5% (w/w) BRB diet to treat the reflux esophagitis and esophageal cancer in male rats induced by an esophagoduodenal anastomosis (EDA) surgical procedure. Our data show that a dietary supplementation of freeze-dried BRB and BB at 2.5% (w/w) is not effective in the prevention of reflux-induced esophagitis and esophageal adenocarcinoma in the EDA animal model, even with an increase of cellular antioxidant enzymes such as MnSOD at the early stage of EDA animals^{31;32}. This discrepancy between our findings and Stoner et al's could be the BRB dosing in the animal diets, for which we used 2.5% (w/w) BRB diet but 5% (w/w) BRB by Stoner³⁰. Therefore, in this study, we use a higher dosage of BRB to treat the animals by oral gavage. It is intriguing but not surprising that the insoluble fraction (residue fraction) is approximately equally as effective as the anthocyanin contained fraction. The residue fraction must contain multiple compounds with anti-inflammation, anti-oxidative stress, and chemo-preventive activities. For example, ellagitannins, has been demonstrated as a chemo-preventive compound in residue fraction³³. We analyzed the ingredients in by GC×GC/TOF-MS, and found that many ingredients are considered as biological active compounds. These ingredients include protocatechuic acid, caffeic acid, ferulic acid, p-coumaric acid, cinnamic acid and p-hydroxybenzoic acid etc (see the supplemental figure).

In conclusion, BRB can act as an effective hepatoprotective agent *via* preserving the activity of MnSOD in the liver. BRB contains multiple bioactive compounds while adding potential advantages: multiple targets effects against the chronic liver injury thereby preventing the carcinogenesis transformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

funding for this research was provided from the Division of Surgical Oncology

Reference List

1. El Serag HB. Hepatocellular Carcinoma. N Engl J Med. 2011; 365:1118–1127. [PubMed: 21992124]
2. El Serag HB. Epidemiology of Viral Hepatitis and Hepatocellular Carcinoma. Gastroenterology. 2012; 142:1264–1273. [PubMed: 22537432]
3. Ivanov AV, Bartosch B, Smirnova OA, Isagulians MG, Kochetkov SN. HCV and Oxidative Stress in the Liver. Viruses. 2013; 5:439–469. [PubMed: 23358390]
4. Min BY, Kim NY, Jang ES, Shin CM, Lee SH, et al. Ethanol Potentiates Hepatitis B Virus Replication Through Oxidative Stress-Dependent and -Independent Transcriptional Activation. Biochem Biophys Res Commun. 2013; 431:92–97. [PubMed: 23274499]

5. Malaguarnera G, Cataudella E, Giordano M, Nunnari G, Chisari G, et al. Toxic Hepatitis in Occupational Exposure to Solvents. *World J Gastroenterol.* 2012; 18:2756–2766. [PubMed: 22719183]
6. Zhu H, Jia Z, Misra H, Li YR. Oxidative Stress and Redox Signaling Mechanisms of Alcoholic Liver Disease: Updated Experimental and Clinical Evidence. *J Dig Dis.* 2012; 13:133–142. [PubMed: 22356308]
7. Xiao J, Ho CT, Liong EC, Nanji AA, Leung TM, et al. Epigallocatechin Gallate Attenuates Fibrosis, Oxidative Stress, and Inflammation in Non-Alcoholic Fatty Liver Disease Rat Model Through TGF/SMAD, PI3 K/Akt/FoxO1, and NF-Kappa B Pathways. *Eur J Nutr.* 2013
8. Tanaka S, Miyanishi K, Kobune M, Kawano Y, Hoki T, et al. Increased Hepatic Oxidative DNA Damage in Patients With Nonalcoholic Steatohepatitis Who Develop Hepatocellular Carcinoma. *J Gastroenterol.* 2013; 48:19. [PubMed: 24203292]
9. Choi J. Oxidative Stress, Endogenous Antioxidants, Alcohol, and Hepatitis C: Pathogenic Interactions and Therapeutic Considerations. *Free Radic Biol Med.* 2012; 52:1135–1150. [PubMed: 22306508]
10. Levent G, Ali A, Ahmet A, Polat EC, Aytac C, et al. Oxidative Stress and Antioxidant Defense in Patients With Chronic Hepatitis C Patients Before and After Pegylated Interferon Alfa-2b Plus Ribavirin Therapy. *J Transl Med.* 2006; 4:25. [PubMed: 16425255]
11. Reddy L, Odhav B, Bhoola KD. Natural Products for Cancer Prevention: a Global Perspective. *Pharmacol Ther.* 2003; 99:1–13. [PubMed: 12804695]
12. Stoner GD, Wang LS, Zikri N, Chen T, Hecht SS, et al. Cancer Prevention With Freeze-Dried Berries and Berry Components. *Semin Cancer Biol.* 2007; 17:403–410. [PubMed: 17574861]
13. Madhusoodhanan R, Natarajan M, Singh JV, Jamgade A, Awasthi V, et al. Effect of Black Raspberry Extract in Inhibiting NF-kappa B Dependent Radioprotection in Human Breast Cancer Cells. *Nutr Cancer.* 2010; 62:93–104. [PubMed: 20043264]
14. Bi X, Fang W, Wang LS, Stoner GD, Yang W. Black Raspberries Inhibit Intestinal Tumorigenesis in Apc1638+/- and Muc2-/- Mouse Models of Colorectal Cancer. *Cancer Prev Res (Phila).* 2010; 3:1443–1450. [PubMed: 21045162]
15. Hecht SS, Huang C, Stoner GD, Li J, Kenney PM, et al. Identification of Cyanidin Glycosides As Constituents of Freeze-Dried Black Raspberries Which Inhibit Anti-Benzo[a]Pyrene-7,8-Diol-9,10-Epoxy Induced NF-kappaB and AP-1 Activity. *Carcinogenesis.* 2006; 27:1617–1626. [PubMed: 16522666]
16. Stoner GD. Foodstuffs for Preventing Cancer: the Preclinical and Clinical Development of Berries. *Cancer Prev Res (Phila).* 2009; 2:187–194. [PubMed: 19258544]
17. Seeram NP. Berry Fruits for Cancer Prevention: Current Status and Future Prospects. *J Agric Food Chem.* 2008; 56:630–635. [PubMed: 18211019]
18. Chang CP, Yang MC, Liu HS, Lin YS, Lei HY. Concanavalin A Induces Autophagy in Hepatoma Cells and Has a Therapeutic Effect in a Murine in Situ Hepatoma Model. *Hepatology.* 2007; 45:286–296. [PubMed: 17256764]
19. Montrose DC, Horelik NA, Madigan JP, Stoner GD, Wang LSW, et al. Anti-Inflammatory Effects of Freeze-Dried Black Raspberry Powder in Ulcerative Colitis. *Carcinogenesis.* 2011; 32:343–350. [PubMed: 21098643]
20. Sun Q, Gao W, Loughran P, Shapiro R, Fan J, et al. Caspase-1 Activation Is Protective Against Hepatocyte Cell Death by Up-Regulating Beclin1 and Mitochondrial Autophagy in the Setting of Redox Stress. *J Biol Chem.* 2013
21. Tudek B, Speina E. Oxidatively Damaged DNA and Its Repair in Colon Carcinogenesis. *Mutat Res.* 2012; 736:82–92. [PubMed: 22561673]
22. Aiyer HS, Kichambare S, Gupta RC. Prevention of Oxidative DNA Damage by Bioactive Berry Components. *Nutr Cancer.* 2008; 60 (Suppl 1):36–42. 10.1080/01635580802398448 [PubMed: 19003579]
23. Tiegs G, Hentschel J, Wendel A. A T Cell-Dependent Experimental Liver Injury in Mice Inducible by Concanavalin A. *J Clin Invest.* 1992; 90:196–203. [PubMed: 1634608]

24. Jaruga B, Hong F, Sun R, Radaeva S, Gao B. Crucial Role of IL-4/STAT6 in T Cell-Mediated Hepatitis: Up-Regulating Eotaxins and IL-5 and Recruiting Leukocytes. *J Immunol.* 2003; 171:3233–3244. [PubMed: 12960353]
25. Gantner F, Leist M, Lohse AW, Germann PG, Tiegs G. Concanavalin A-Induced T-Cell-Mediated Hepatic Injury in Mice: the Role of Tumor Necrosis Factor. *Hepatology.* 1995; 21:190–198. [PubMed: 7806154]
26. Mizuhara H, Uno M, Seki N, Yamashita M, Yamaoka M, et al. Critical Involvement of Interferon Gamma in the Pathogenesis of T-Cell Activation-Associated Hepatitis and Regulatory Mechanisms of Interleukin-6 for the Manifestations of Hepatitis. *Hepatology.* 1996; 23:1608–1615. [PubMed: 8675184]
27. Dasgupta J, Subbaram S, Connor KM, Rodriguez AM, Tirosh O, et al. Manganese Superoxide Dismutase Protects From TNF-Alpha-Induced Apoptosis by Increasing the Steady-State Production of H₂O₂. *Antioxid Redox Signal.* 2006; 8:1295–1305. [PubMed: 16910777]
28. Pardo M, Tirosh O. Protective Signalling Effect of Manganese Superoxide Dismutase in Hypoxia-Reoxygenation of Hepatocytes. *Free Radic Res.* 2009; 43:1225–1239. [PubMed: 19905985]
29. Li Y, Reuter N, Li XS, Martin RC. Manganese Superoxide Dismutase Chemoprevention in a Murine Hepatitis Associated Injury Model. *Journal of Cancer Therapy.* 2011; 2:431–440.
30. Wang LS, Hecht SS, Carmella SG, Yu N, Larue B, et al. Anthocyanins in Black Raspberries Prevent Esophageal Tumors in Rats. *Cancer Prev Res (Phila).* 2009; 2:84–93. [PubMed: 19139022]
31. Aiyer HS, Li Y, Liu QH, Reuter N, Martin RC. Dietary Freeze-Dried Black Raspberry's Effect on Cellular Antioxidant Status During Reflux-Induced Esophagitis in Rats. *Nutrition.* 2011; 27:182–187. [PubMed: 20538426]
32. Aiyer HS, Li Y, Losso JN, Gao C, Schiffman SC, et al. Effect of Freeze-Dried Berries on the Development of Reflux-Induced Esophageal Adenocarcinoma. *Nutr Cancer.* 2011; 63:1256–1262. [PubMed: 22043833]
33. Ross HA, McDougall GJ, Stewart D. Antiproliferative Activity Is Predominantly Associated With Ellagitannins in Raspberry Extracts. *Phytochemistry.* 2007; 68:218–228. [PubMed: 17126865]

Figure 1. Effect of BRB extracts on histological changes and LAT in Con A mice

A: Representative histology of Con A induced liver injury and BRB extracts protection, Hematoxylin and eosin staining ($\times 200$). **B:** Histopathology scores including inflammatory infiltration and hepatocyte death were graded on a severity scale from 0 to 3 (0 = no lesion, 1 = mild, 2 = moderate, 3 = severe). **C:** Serum ALT levels in Con A liver injury mice and BRB extracts mice. UT: saline control group; A: BRB-A; B: BRB-B; C: BRB-C. The data presented by mean $\pm$ SD, * $P < 0.05$ compared to Con A group.

Figure 2. Effect of BRB extracts on apoptosis, lipid peroxidation and 8-OH-dG in Con A mice
A: Representative apoptotic regions. Con A induced liver injury and BRB extracts protection by TUNEL assay. **B:** Apoptotic index by the percentage of TUNEL positive cells over total cells. **C:** lipid peroxidation by (TBARS) Assay to measure the MDA levels in the hepatic tissues. **D:** 8-OH-dG level by immunohistochemical staining and computer image-analysis. UT: saline control group; A: BRB-A; B: BRB-B; C: BRB-C; Th: threshold. The data presented by mean $\pm$ SD, *P < 0.05 compared to Con A group.

Figure 3. Effect of BRB extracts on SODs, Glutathione and Catalase in Con A mice

A: SODs enzymatic activities. **B:** GSH concentration. **B:** Catalase enzymatic activities UT: saline control group; A: BRB-A; B: BRB-B; C: BRB-C. The data presented by mean $\pm$ SD, *P < 0.05 compared to Con A group.

Figure 4. Effect of BRB extracts on hepatocytes challenged by ischemia/reoxygenation
A: Cell viability by MTT assay in the hepatocytes challenged by ischemia/reoxygenation. **B:** MnSODs enzymatic activities in the hepatocytes challenged by ischemia/reoxygenation. **C:** MnSOD protein levels by Western blot analysis. UT: saline control group; I/R: ischemia/reoxygenation; A: BRB-A; B: BRB-B; C: BRB-C; M: MnTBAP. The data presented by mean $\pm$ SD, *P < 0.05 compared to ischemia/reoxygenation group.