

## Black Raspberry Extract and Fractions Contain Angiogenesis Inhibitors

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Targeted therapies, such as agents that inhibit angiogenesis, offer hope as complementary agents in cancer therapy. Angiogenesis-inhibiting agents have the potential for inhibiting tumor growth and limiting the dissemination of metastasis, thus keeping cancers in a static growth state for prolonged periods. Black raspberry (*Rubus occidentalis*) extract was discovered to be antiangiogenic (0.1% w/v) in a novel human tissue-based in vitro fibrin clot angiogenesis assay. Assay-guided fractionation of a crude black raspberry extract resulted in a highly potent antiangiogenic fraction that accounted for only 1% of the fresh weight of whole black raspberries. At 0.075% (w/v), the active fraction completely inhibited angiogenic initiation and angiogenic vessel growth. Further subfractionation of this active fraction revealed the coexistence of multiple antiangiogenic compounds, one of which has been identified as gallic acid. However, the individual subfractions did not outperform the active whole fraction. These findings suggest that an active black raspberry fraction may be a promising complementary cancer therapy. It is natural and potent enough for manageable dosing regimens. These extracts contain multiple active ingredients that may be additive or synergistic in their antiangiogenic effects. These observations warrant further investigations in animals and human trials.

**KEYWORDS:** Angiogenesis; black raspberries; extraction; fingerprinting; fractionation; initiation; human tissue; neovessels; standardization; synergism

### INTRODUCTION

Angiogenesis is the process by which new blood vessels grow. In the adult, except for a few physiological processes such as menses, wound healing, and placental formation, all angiogenic processes are pathologic (1–3). By blocking the development of new blood vessels, one hopes to cut off the tumor's supply of oxygen and nutrients and, therefore, its growth and spread to other parts of the body (4–6). The working hypothesis in the medical research community is that if angiogenesis can be prevented or reversed, angiogenesis-dependent conditions and diseases can also be prevented or reversed. The three ways that angiogenic-modulating compounds can interfere with an angiogenic process are by preventing the initiation of angiogenesis (the angiogenic switch); by inhibiting neovessel growth if it has already begun; or by inhibiting both steps in the angiogenic cascade. Because angiogenesis inhibitors target new blood vessel

formation, using them in cancer treatment is classified as a targeted therapy. Solid tumors cannot grow beyond 1–2 mm<sup>3</sup> without inducing the formation of new blood vessels to supply the tumor's nutritional needs (5). Drug resistance is a major problem with chemotherapeutic agents because most cancer cells are genetically unstable, prone to mutations, and therefore more likely to produce drug resistant cells. Because antiangiogenic drugs target normal endothelial cells that are genetically stable, drug resistance may not develop (7–9). Antiangiogenic therapy may prove complementary to antineoplastic therapy aimed directly at tumor cells (e.g., chemotherapeutic agents). Because chemotherapy and antiangiogenic therapy have different cellular targets, there is hope that the combination will prove more effective than either therapy alone. In fact, patients with colon cancer in a recent clinical trial improved survival when they received Avastin, an antiangiogenic drug, along with topotecan, standard chemotherapy (10).

In the screening of botanical samples, particularly food items, for antiangiogenic activity, we found that that a black raspberry (*Rubus occidentalis*) extract had some, but not strong, inhibitory activities over human angiogenesis in vitro in a fibrin thrombin-based assay. Black raspberry is one of approximately 250 species

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in the *Rubus* genus of the Rosaceae (rose) family. Black raspberry is a perennial shrub native to North America. The berries are known to be rich in anthocyanins (11), pectin, fruit acids, ellagic acid, and vitamins A, B1, and C. Six common berries were found to be potent chemopreventive agents via inhibition of angiogenesis (12). However, the active ingredient(s) in these berries have not yet been identified. We hypothesized that this berry contains antiangiogenic ingredients; that fractions of this berry extract are more potent than the crude extract; that multiple active ingredients coexist; and that these fractions can be concentrated into a highly effective therapy. To test these hypotheses, we conducted a series of bioassay-guided fractionations under the direction of human tissue-based angiogenesis assays. These preliminary characterizations provide a framework for achieving standardization for this berry and its active components, which is required for future evaluations *in vivo*.

## MATERIALS AND METHODS

**Preparation of Berry Extract.** Frozen whole black raspberries (WBRs) were obtained from a commercial grower in Oregon. The whole berries (908 g) were extracted in deionized water at 1:15 w/v ratio, and the liquid was brought to boil for 30 min in a heating mantle. The supernatant was separated from the residue first by centrifugation at 2600g and subsequently by a filtration system consisting of 20, 1, and 0.45  $\mu\text{m}$  filter units in sequence (Ultrafilter International, Haan, Germany). The liquid extract was then concentrated under reduced pressure before freeze drying to crude powder (WBR-C), which accounted for 15% (136 g) of the weight of the WBRs. The crude extract powder was frozen at  $-20\text{ }^{\circ}\text{C}$  and stored in a sealed container for later bioassay-guided fractionation using angiogenesis assays.

**Instrumentation.** Chemical analysis and fingerprinting of the berry extracts were performed on a Delta 600 high-performance liquid chromatography (HPLC) system (Waters Co., Milford, MA), consisting of a solvent delivery pump unit, a 717 plus autosampler, a model 2996 UV/vis photodiode array detector at 190–800 nm, and a model 2420 evaporative light-scattering detector. The system was computer controlled, and the data were analyzed with the Empower software system. The HPLC system was used for both analytical and preparative separations. The analytical separation was performed with a 150 mm  $\times$  4.6 mm i.d., 5  $\mu\text{m}$  Symmetry C18 column and the semipreparative separation was performed with a 150 mm  $\times$  19 mm, 7  $\mu\text{m}$  Atlantis C18 ODS column (Waters Co.). The mobile phase consisted of methanol (A) and aqueous acetic acid (0.15%) (B) and was run in the following scheme: 0–12 min, 5% A; 13–26 min, 5–100% A; 27–30 min, 100% A; and 31–45 min, 5% A.

**Fractionation of Active Extracts.** The crude extract WBR-C powder (100 g) was further subjected to a series of column chromatography steps, first by using a molecular sieve-based separation method (Supelco, Bellefonte, PA). The column was first equilibrated with water. WBR-C powder was dissolved in water at a 1:8 ratio (w/v). After the liquid WBR-C sample was loaded, four fractions were eluted with aqueous ethanol, 1000 mL each (0, 20, 50, and 95% ethanol), to obtain WBR-00, WBR-20, WBR-50, and WBR-95. The liquid fractions were then evaporated to remove ethanol and subsequently freeze-dried and analyzed on HPLC to develop chromatograms as described above. The freeze-dried fractions, WBR-00, WBR-20, WBR-50, and WBR-95, accounted for 39, 14, 3.4, and 6.5%, of the crude extract by weight, respectively. The rest of the percentage (approximately 37%) was not unrecoverable during the fractionation process.

The active fraction WBR-95 (2.0 g) was further fractionated on a polarity-based flash chromatography (ISCO CombiFlash Graduate, Lincoln, Nebraska). The aqueous sample was loaded onto a 20 g C18 column and eluted sequentially with 125 mL of water and 20, 50, and 100% of aqueous methanol (MeOH) to yield four subfractions. The organic liquid fractions were evaporated under reduced pressure to remove organic solvent, and the resulting aqueous fractions were freeze-dried to obtain powdered subfractions, WBR-95A, WBR-95B, WBR-95C, and WBR-95D, with extraction rates of 40, 39, 13, and 4.5% of the WBR-95, respectively.

On the basis of the UV analysis of each peak present in the active fraction WBR-95, the presence of gallic acid was suspected. Using the same mobile phase conditions described in above instrumentation section, isolation of gallic acid was conducted on a 150 mm  $\times$  19 mm, 7  $\mu\text{m}$  Atlantis semipreparative column with an isocratic elution using absolute MeOH:0.15% acetic acid water (5:95) at a flow rate of 5.0 mL/min. The structure of the purified compound was identified by NMR and MS.  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR were performed on Bruker DPX-400 spectrometer, and MS was measured on Varian Saturn 2200 GC/MS.

**In Vitro Human Tissue-Based Angiogenesis Assay.** Before coculturing with human tissues, frozen extracts were brought to room temperature and were reconstituted with a human tissue culture medium. The reconstituted extract was sterilized with 0.22  $\mu\text{m}$  filters (Millex, Millipore, Billerica, MA) under sterile hood before angiogenesis assays. The sterile aqueous extract was used to treat human placental vein tissues prepared in 96 well plates for up to 15 days.

**Tissue Source and Preparation.** A human placental vein angiogenesis model (HPVAM) was used in all of the tests. Tissue preparation followed previously published methods (13–18). Discarded human placentas were obtained anonymously with a prior approved protocol of the LSUHSC Institutional Review Board. The placental veins were dissected free from the placenta and adventitial tissue. The trimmed vein segments were opened longitudinally to produce a flat film of venous tissue of full thickness. Vein disks (2 mm diameter) were created with a sterile skin punch (Sklar Instruments, West Chester, PA). The disks were placed into wells of a standard 96 well plate (Corning Inc., Corning, NY). The vein disk harvest was completed within 3 h of delivery to optimize endothelial cell viability. Vein disks from a single placenta were distributed randomly among all treatment groups to minimize the effect of vein-to-vein variation. Each well was preloaded with 2  $\mu\text{L}$  of bovine thrombin solution at 400 U/mL and allowed to evaporate to dryness before use. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated.

**Tissue Culture.** Following the placement of the 2 mm vein disk in the bottom of each thrombin-containing well, the disk was covered with 100  $\mu\text{L}$  of a clot-forming medium, comprised of 3 mg/mL fibrinogen and 0.5%  $\Sigma$ -amino caproic acid dissolved in HPVAM medium. The HPVAM medium was made of Medium 199 (Invitrogen Corporation, Carlsbad, CA), an antibiotic/antimycotic solution [100 U/mL penicillin, 100 U/mL streptomycin sulfate, and 0.25  $\mu\text{g}/\text{mL}$  amphotericin  $\beta$  (Invitrogen Corporation)]. The mixture was allowed to clot by incubating it in 5%  $\text{CO}_2$ , 95% air at 37  $^{\circ}\text{C}$  in a humidified incubator for 1 h. After the medium-containing placental disks had clotted, the vein-containing clot was overlaid with 100  $\mu\text{L}$  of HPVAM medium containing 20% fetal bovine serum (Invitrogen Corporation). The total well volume was 200  $\mu\text{L}$ .

**Treatments.** Vein disks were treated by the addition of the plant extract of interest to the serum-containing medium liquid overlay. Treatments occurred every other day for the duration of the experiment, usually 14 days, by replacement of fresh media (100  $\mu\text{L}$ ) containing the appropriate plant extract. Generally, 30 wells per treatment group were established ( $n = 30$ ). A heparin-steroid (H/S) treatment group [300  $\mu\text{L}/\text{mL}$  heparin, 180 U/mg (Sigma), and 350  $\mu\text{g}/\text{mL}$ , hydrocortisone phosphate (Spectrum Chemical, New Brunswick)] was added in most experiments to demonstrate inhibition of angiogenesis as previously reported (15). Serum-containing medium served as the untreated control.

**Measurements.** Angiogenesis was evaluated using an inverted microscope at 10 $\times$  magnification with a standardized reference grid. Scoring was performed using a standard blinded format: extracts/fractions were coded, and no specific knowledge of the extract/fraction or its concentration was known to the person who performed the scoring. Angiogenesis was evaluated using two criteria: initiation of angiogenesis (%I) and the extent of neovessel growth (angiogenic index, AI) as outlined previously (13, 15–18). Neovessel growth was evaluated every other day.

Initiation of an angiogenic response was defined as the development of three or more vessel sprouts around the periphery of the vein disk. Initiation (%I) is expressed as the percent of the total wells plated that develop an angiogenic response. Unless stated otherwise, 30 wells were prepared for each treatment group, and so %I represents the fraction

of those wells that initiated an angiogenic response. As a ratio, this value does not produce a standard deviation. Generally, one experiment (placenta) was performed with each set of extracts/preparations. Previous experiments showed that initiation occurs in 50–95% of the wells, usually 4–6 days after establishment of the clots.

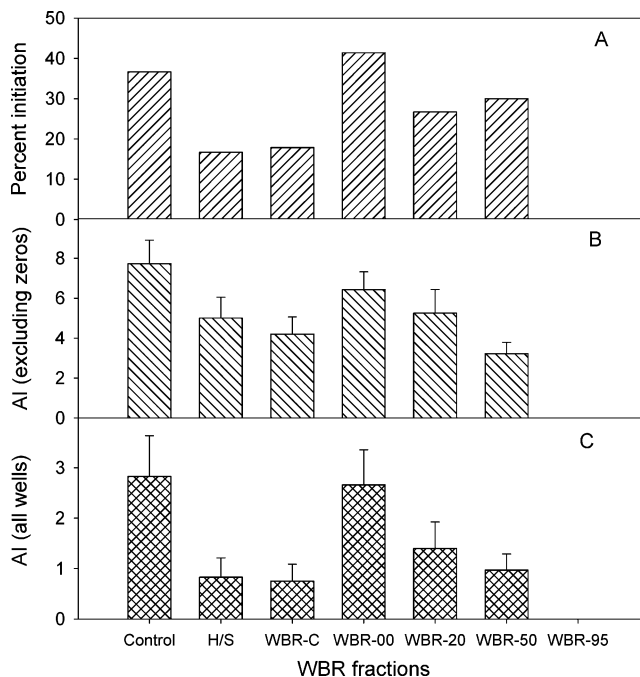
The AI is defined using a semiquantitative visual rating system developed and validated in our laboratory. Each disk was visually rated for the development of vessel sprouting in each of four quadrants. Each of the four quadrants for each disk was rated on a 0–4 scale, depending on the number of sprouts (density), the length of sprouts, and the percent of the quadrants' circumference involved with the response. Scores for all four quadrants were summed to express the AI, a numerical rating that could range from 0 to 16. A score of zero indicates no vessel growth in any of the four quadrants, while a score of 16 indicates long, dense angiogenic vessel growth in all four quadrants. For most experiments, the AI is expressed as a mean plus/minus a standard error of the mean for each treatment group (30 wells). The mean AI can be expressed in two forms: by including zero values (all wells) and by excluding zero values. A zero AI indicates that no angiogenic initiation occurred in that disk. This lack of initiation could be due either to the effect of the experimental compound, the insensitivity of the vein disk to stimulation in the culture conditions, or the vein disk not being viable. In previous experiments, we have shown that only a small percent, about 2–3%, of vein disks are not viable when tested for uptake and subsequent conversion of its formazan dye (MTT). The AI with zeros can be thought of as a parameter that evaluates both initiation and growth (AI/combined response), while the AI without zeros is a parameter of pure angiogenic growth (AI/growth).

**Tissue Viability Study.** Following a 14 day assay, embedded tissues were tested for viability as an indication of cytotoxicity. Spent media were aspirated and replaced with 100  $\mu$ L of HPVAM medium containing 15  $\mu$ L of MTT dye solution (Promega). Tissues were incubated for 48 h at 37 °C and 5% CO<sub>2</sub> (number of wells = 10 for both WBR-95 and the control). Wells were then observed microscopically under low power to determine if the MTT had been metabolized to the blue formazan dye. The presence of the formazan dye crystals was scored positive (viable) while the absence was scored negative (not viable). Cell proliferation could not be quantified as is typically done with MTT dye assays due the technical problems associated with the biphasic nature of the culture media used in this model.

**Statistical Analyses.** Analysis of variance with repeat measures was performed on the angiogenic responses using SAS programming (Statistical Analysis System, Cary, NC) for standard tests of significance. Treatment means were separated at  $P \leq 0.05$ .

## RESULTS

**Black Raspberry Extract and Its Fractions Inhibited Human Angiogenesis.** At 0.1% (w/v), treatment with the crude extract WBR-C resulted in a 50% inhibition in the angiogenic response (%I; **Figure 1A**). The WBR-C treatment decreased in the number of wells that initiated an angiogenic response (18%) as compared to untreated control wells (37%) and was similar to the value obtained using H/S (17%), a treatment known to inhibit angiogenesis. Initiation of an angiogenic response in the three fractions, WBR-00, WBR-20, and WBR-50, was less inhibitory (41, 27, and 30%, respectively) than the crude extract WBR-C. Moreover, WBR-00 appeared to stimulate angiogenesis as compared to the untreated controls. However, at the same concentration of 0.1% (w/v), the WBR-95 fraction completely suppressed angiogenic initiation (%I = 0). Neovessel growth in wells that did initiate an angiogenic response (AI/growth, zeros excluded; **Figure 1B**) was also influenced by several of these compounds. The crude extract WBR-C and one of its fractions WBR-50 demonstrated statistically less growth ( $p < 0.05$ ) as compared to control values (AI/growth for WBR-C, WBR-50, and control: 4.2, 3.2, and 7.7, respectively). However, the remaining two fractions, WBR-00 and WBR-20, did not significantly inhibit neovessel growth with AI/growth values of 6.4 and 5.3, respectively. When all wells were included in

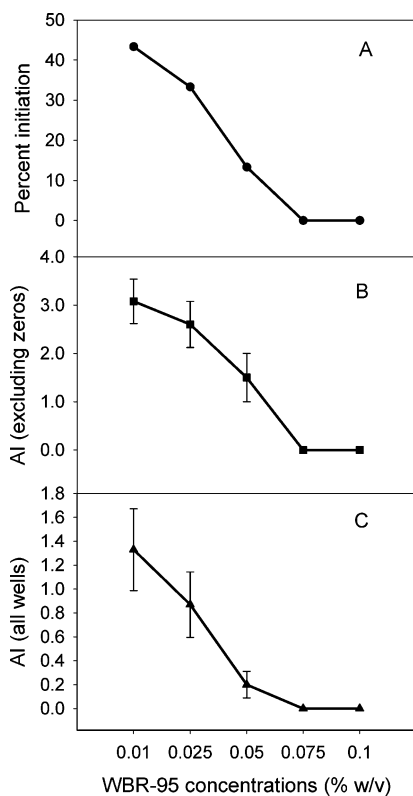


**Figure 1.** Antiangiogenic activities of an aqueous crude extract of black raspberries (WBR-C) and its four fractions tested at 0.1% (w/v) concentrations. (A) Angiogenic initiation; (B) angiogenic vessel growth (wells that did not initiate an angiogenic response excluded); and (C) overall angiogenic response in all wells (wells with zero values included). H/S was used as a standard for inhibition. Vertical bars at each data point represent one standard error of the mean. The data represent one experiment (placenta) with 30 wells each (control, H/S, WBR-20, WBR-50, and WBR-95), 29 wells each (WBR-00), or 28 wells each (WBR-C) at day 14 of culture.

the statistical analysis (AI/combined response, zeros included; **Figure 1C**), treatment with H/S and the crude extract WBR-C significantly inhibited angiogenesis ( $p < 0.05$ ), with identical values of 0.8 as compared to the control value of 2.8. The fraction WBR-50 showed a similar degree of inhibition with a value of 1.0. The AI/combined response for wells treated with WBR-00 and WBR-20 was 2.7 and 1.4, respectively. The complete inhibition of angiogenesis by the WBR-95 fraction was statistically significant.

To determine the antiangiogenic potency of WBR-95 (the most potent fraction isolated from the crude extract WBR-C), a dose–response study was conducted using a 10-fold range in concentration from 0.01 (w/v) to 0.1% (w/v). At 0.075 and 0.1% (w/v), the WBR-95 fraction completely inhibited angiogenesis (%I and AI value of 0). However, linear dose–responses for concentrations of 0.01, 0.025, 0.05, and 0.075% WBR-95 were observed for the initiation of an angiogenic response with values of 43, 33, 13, and 0%, respectively (**Figure 2A**), AI/growth (zeros excluded), with values of 3.1, 2.6, 1.5, and 0, respectively (**Figure 2B**), and AI/combined response with values of 1.3, 0.9, 0.2, and 0 respectively (**Figure 2C**).

To determine if more than one active component existed in the refined WBR-95 extract, a subfractionation was performed, and the potential inhibitory activities were assessed using the HPVAM model. The original WBR-95 fraction inhibited initiation of an angiogenic response to a greater extent than any of the subfractions (A–D) reaching only 15% by day 14 of culture. However, all of the subfractions (A–D) of WBR-95 reduced initiation of angiogenesis by day 14 (30, 30, 45, and 25%, respectively) when compared to the untreated control value of 77% and the H/S inhibitory standard of 55% (**Figure 3A**).

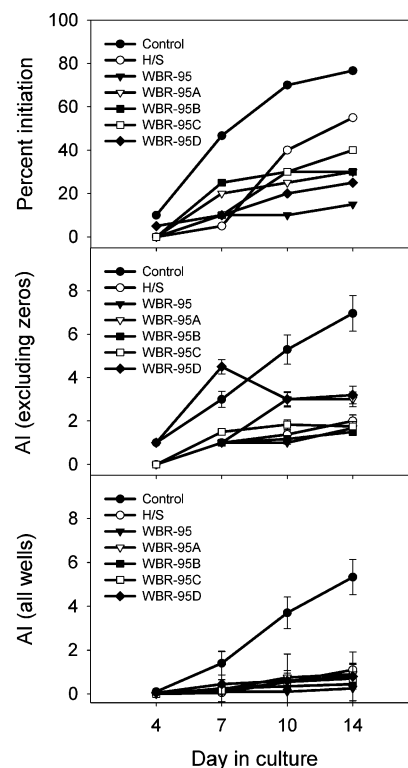


**Figure 2.** Dose–response of the antiangiogenic activities of the active fraction, WBR-95, separated from the aqueous crude extract of black raspberries, WBR-C. (A) Angiogenic initiation; (B) angiogenic vessel growth (wells that did not initiate an angiogenic response excluded); and (C) overall angiogenic response in all wells (wells with zero values included). Vertical bars at each data point represent one standard error of the mean. The data represent one experiment (placenta) with 30 wells for all treatment groups at day 15 of culture.

On those tissues that initiated angiogenic responses, (AI/growth; zeros excluded), the treated wells showed a profound reduction in angiogenic vessel growth on day 14 (**Figure 3B**). The untreated control exhibited a linear growth pattern during the culture period, indicative of healthy and robust growth conditions. Tissues exposed to WBR-95 and its subfractions WBR-95B and WBR-95C displayed very little growth throughout the 14 day culture and provided AI/growth values of 1.67, 1.5, and 1.75 for WBR-95, WBR-95B, and WBR-95C, respectively, which compare with the AI/growth values of 6.95 for control and 2.0 for H/S by day 14 of culture. The remaining two subfractions WBR-95A and WBR-95D were less inhibitory (AI/growth values of 3.0 for WBR-95A and 3.2 for WBR-95D by day 14 of culture). With the combined response (AI, zeros included), the WBR-95 fraction and all of its subfractions showed dramatically reduced angiogenesis as compared to the untreated control during the culture period (**Figure 3C**). Notably, none of the subfractions out-performed the fraction WBR-95, from which they originated at any point in time throughout the 14 days in culture.

**Refining the Antiangiogenic Fraction WBR-95.** The potent antiangiogenic fraction isolated as WBR-95 accounted for 6 wt % of the crude extract WBR-C or 1 wt % of the fresh berries. The majority (94%) of WBR extract (WBR-C) either did not show or showed very weak inhibitory activity in the human angiogenesis assay.

**Chemical Fingerprints of the Active Antiangiogenic Fraction.** WBR-C is a complex mixture of compounds, as seen in

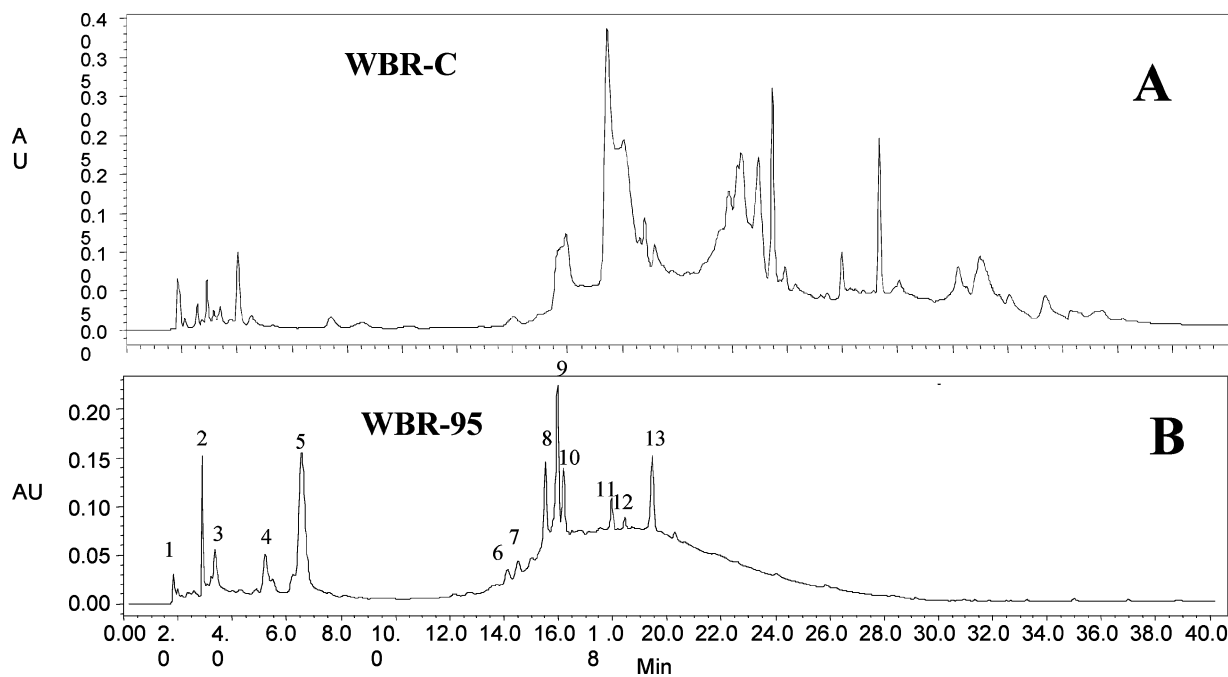


**Figure 3.** Antiangiogenic activities of the refined antiangiogenic fraction WBR-95 and its four subfractions tested at 0.05% (w/v) concentrations. (A) Angiogenic initiation; (B) angiogenic vessel growth (wells that did not initiate an angiogenic response excluded); and (C) overall angiogenic response in all wells (wells with zero values included). H/S was used as a standard for inhibition. Vertical bars at each data point represent one standard error of the mean. The data represent one experiment (placenta) with 30 wells for the control and 20 wells for all other treatment groups through day 14 of culture.

its HPLC chemical fingerprint (**Figure 4A**). There are at least 37 identifiable components based on UV absorption patterns. In reality, there are probably more than 37 compounds, since some peaks may be composed of more than one compound, others may have low UV absorption, and yet others may be in extremely low concentrations that are easily hidden in the baseline. However, the chemical fingerprint proved to be extremely useful in practice and as a method to validate new test samples with authentic black raspberry sample.

The WBR-95 antiangiogenic fraction, however, was much simpler in chemical composition (**Figure 4B**). Chemical fingerprinting revealed 13 major components, a 65% reduction from the 37 major components in the crude extract as shown in **Figure 4A**.

**Gallic Acid Is One of the Active Compounds in the Refined Black Raspberry Extract WBR-95.** Peak 4 in group 2 was isolated and purified. Structural elucidation identified this pure compound as gallic acid [ $^1\text{H NMR}$  ( $\text{CD}_3\text{OD}$ )  $\delta$ : 7.01 (2H, s), 4.89 (OH).  $^{13}\text{C NMR}$  ( $\text{CD}_3\text{OD}$ )  $\delta$ : 170.37, 146.36, 139.57, 121.94, 110.31. EI-MS  $m/z$  170 ( $\text{M}^+$ )]. Comparison was made with a standard gallic acid compound purchased from Sigma Chemical Company, and the two structures were identical. Therefore, commercial gallic acid was tested for its inhibition of angiogenesis in the HPVAM assay. Control values in this experiment were consistent with those observed previously, with 100% of the wells initiating an angiogenic response, an AI/growth (zeros excluded) of 6.1, and an AI/combined response (zeros included) of 3.72. At  $10^{-3}$  M, gallic acid completely



**Figure 4.** Chromatograms of black raspberry extracts developed on a high performance liquid chromatography (HPLC) at a wavelength 254 nm. (A) Black raspberry crude extract (WBR-C); (B) the active fraction (WBR-95).

inhibited angiogenic initiation and neovessel growth (data not shown). Quantitative analysis on HPLC indicates that the active WBR-95 fraction contained 0.42% (w/w) gallic acid.

## DISCUSSION

Traditionally, the angiogenic response has been tested using well-established animal or cell culture models. These include the chicken chorioallantoic membrane model and the corneal assay, both of which are animal models; thus, the applicability of the resultant data to the human condition has always been suspect (19, 20). In an effort to avoid interspecies variation, a number of authors have used human umbilical vein endothelial cells (HUVECs) in proliferation assays, migration assays, or tube formation assays. Unfortunately, the umbilical vein endothelial cells used in these assays have already been introduced into monolayer culture and are proliferating in culture. Thus, the “angiogenic switch” described by Folkman and Hanahan (21, 22) has already occurred. Freeman and Parish (23) and Parish et al. (24) demonstrated that the human placental veins could be harvested, opened longitudinally, and full-thickness venous fragments embedded in a three-dimensional fibrin–thrombin clot. These vascular explants, over time, developed an angiogenic neovessel response, which grew in a progressive fashion over 2–3 weeks in culture. This HPVAM has been utilized to test a variety of known angiogenesis inhibitors and experimental compounds (7) including: heparin, hydrocortisone, external beam radiation, suramin analogues, endostatin, angiostatin, and the tubulin inhibitor, epothilone B. Clearly, the HPVAM has significant advantages over HUVEC-based models. This model allows an investigator to study several apparently independent phases of the human angiogenic response. First, the angiogenic switch from resting to proliferative vascular endothelium can be determined by calculating the number of wells that develop an angiogenic response as a percent of the total wells plated (% initiation). In contrast, once a well has initiated an angiogenic response, sequential grading of the length, density, and the percent of the vessel disk’s circumference involved with neovessels can yield a functional “AI” that

indicates neovessel growth. Certain treatments have no effect on initiation (25), while other treatments affect only the initiation step of the angiogenic response (19). Other treatments affect initiation and the subsequent growth of neovessels in a roughly equal proportion (14).

This HPVAM model established that black raspberry extract contains angiogenic inhibitors that affected both the initiation step and the subsequent neovessel growth step of the angiogenic response. The black raspberry extract inhibited angiogenic initiation, preventing the angiogenic switch from a resting to proliferating endothelium. The black raspberry extract was also a potent inhibitor to neovessel growth once an angiogenic initiation had taken place. We have used commercially grown black raspberries obtained from a single source for these studies. Although there is always a possibility that a contaminant (i.e., fungal or other microbial, pesticide, or other chemical) may account for some of the observed effects, that possibility is very small. First, an active compound, gallic acid, was isolated and elucidated from these berries and was shown partially responsible for the antiangiogenic activity. Other yet-to-be elucidated antiangiogenic compounds from these berry extracts are being sought. Second, the antiangiogenic activity of black raspberries was found by other investigators who used different sources from the one used in this study (12). Black raspberry is a fruit in our diet. For years, U.S. Department of Agriculture inspectors have used black raspberry juice as a natural “ink” to stamp commercial meat products, indicative of safety entering the human food chain. Our discovery of the antiangiogenic property of black raspberries and identification of gallic acid as an antiangiogenic ingredient further expands our knowledge about this bioactive food. Recently, black raspberries have been shown to possess antioxidant properties (26, 27). Strong antioxidant activity corresponds to the high anthocyanin and phenolic content of this fruit (11, 28). Black raspberry extract was found to inhibit tumor development in rodents, possibly by impairing signal transduction pathways leading to activation of activated protein 1 and nuclear factor  $\kappa$ B (29) and by inhibiting the activity of cyclooxygenase (30). Others studies suggest that a component-

(s) in black raspberry influences the metabolism of *N*-nitrosomethylbenzylamine (31). This chemopreventive effect has primarily been attributed to the ellagic acid in black raspberries, which has been shown to inhibit cancers induced in rodents by several carcinogens (32). Xue et al. (33) assessed the chemopreventive activity of ellagic acid and black raspberry fractions in a Syrian hamster embryo cell transformation model. They found that ellagic acid and a methanol fraction of the black raspberry produced a dose-dependent decrease in transformation, possibly through interfering with the uptake, activation, and/or detoxification of the carcinogenic benzo[*a*]pyrene and/or the intervention in DNA binding and DNA repair. Six common berries including raspberry seeds (the current study used WBRs) were shown to inhibit angiogenesis in a matrigel assay and significantly inhibited TNF- $\alpha$ -induced VEGF expression by the human keratinocytes. These activities plus the strong antioxidant property of these berries were thus postulated to be potent natural chemopreventive agents (12). Further studies of the blueberry and six berry mix confirmed their antiangiogenic properties in an *in vivo* model of hemangioma when berry powder-treated endothelioma cells were injected into mice (34). Bagchi et al. (35) reviewed the bioactivities displayed by the edible berries and indicated that they possessed antiangiogenic, antioxidant, and anticarcinogenic properties. Anthocyanins may be partially responsible for these activities. However, there is no literature to confirm that anthocyanins in these berries are responsible for antiangiogenic activities; neither are the structures elucidated. The present study confirmed that gallic acid is an antiangiogenic compound partially responsible for the antiangiogenic activity of black raspberry and that there is another active compound(s) in the black raspberry responsible for the antiangiogenic activity. Whether these multiple active compounds are exerting an antiangiogenic effect through additive and/or synergistic approach is worth investigating when the structures are elucidated.

We showed that a refined black raspberry extract, WBR-95, is more potent than the crude extract in the inhibition of angiogenesis. However, none of the individual subfractions of WBR-95 outperformed the originating WBR-95, which may suggest that key components are additive or synergistic in their ability to inhibit angiogenesis. Furthermore, this explains that further separation of this fraction would result in diminished antiangiogenic activity. Chemically characterizing this refined WBR-95 extract followed by standardization of key active chemical markers could provide a reliable antiangiogenic extract for clinical testing.

The active antioxidant compounds in black raspberry are bioavailable through the diet (36). However, the absorption of the bioactive components might be small and thus might fall short of reaching therapeutic doses. As a matter of fact, dietary intervention often fails in clinical studies (37), probably due to the low levels and huge variability of the unidentified active compounds in the berry diet tested. This strongly suggests that an extract must be standardized and contain a therapeutic concentration of the active compound(s) to produce a consistent clinical effect.

Our refining process has resulted in a highly refined black raspberry extract, which is a small (1 wt %) fraction of the fresh, whole raspberries. This concentration of the extract is extremely meaningful therapeutically because it allows the antiangiogenic extract to be dosed in amounts more likely to be therapeutically effective. In a clinical setting, underdosing is most likely when the active ingredients in the extract are low and large doses are infeasible (e.g., 100 capsules/day). A botanical fraction rich in

antiangiogenic ingredients could achieve a concentration that would allow a feasible dosing regimen. The refined black raspberry extract translates into the approximate equivalence of 1 g of WBR-95 extract to 100 g of WBRs. It is obvious that this refined extract offers a great advantage in designing a dosing regimen for therapeutic/preventative trials.

This study presents preliminary evidence that the black raspberry contains compounds that possess antiangiogenic effects. Bioassay-guided fractionations resulted in a highly refined active WBR-95 extract accounted for only 1 wt % of WBRs. This refined extract contained multiple components, one of which is gallic acid. None of the individual subfractions outperformed their originating extract WBR-95, indicating possible additive/synergistic effects of individual active components. If the active ingredients were additive or synergistic in their antiangiogenic effect, this interaction would theoretically lower the required therapeutic dose allowing a practical dosing regimen to be designed for human clinical trials of this extract. While this is a preliminary characterization of black raspberry extracts, it paves the ways for determining the chemical structure of other active compounds in the black raspberry subfractions and for conducting *in vivo* efficacy study on tumor-bearing rodent models, which is under way.

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