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Antimutagenic Activity of Berry Extracts

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ABSTRACT Plants are proven sources of useful anti-tumor and chemopreventative compounds. Hence, identification of phytochemicals useful in dietary prevention and intervention of cancer is of paramount importance. The initial step in the formation of cancer is damage to the genome of a somatic cell producing a mutation in an oncogene or a tumor-suppressor gene. Fresh juices and organic solvent extracts from the fruits of strawberry, blueberry, and raspberry were evaluated for their ability to inhibit the production of mutations by the direct-acting mutagen methyl methanesulfonate and the metabolically activated carcinogen benzo[*a*]pyrene. Juice from strawberry, blueberry, and raspberry fruit significantly inhibited mutagenesis caused by both carcinogens. Ethanol extracts from freeze-dried fruits of strawberry cultivars (Sweet Charlie and Carlsbad) and blueberry cultivars (Tifblue and Premier) were also tested. Of these, the hydrolyzable tannin-containing fraction from Sweet Charlie strawberries was most effective at inhibiting mutations.

KEY WORDS: • ***anticancer*** • ***ellagic acid*** • ***ellagitannin*** • ***small fruits***

INTRODUCTION

CANCER CLAIMS THE LIVES of nearly 7 million people worldwide every year. An important approach toward reducing cancer incidence and mortality is through dietary intervention and prevention. An abundance of data indicates that diets high in fruits and vegetables are effective in protecting humans from cancer. Research on the effects of diet on cancer has demonstrated nearly 1,000 phytochemicals with cancer prevention activity, but fewer than 25 compounds have been tested in clinical trials. *In vitro* data have been collected to support tumor-inhibitory properties of phytohormones, apoptosis-inducing compounds, and substances that induce detoxification of potential mutagens.[1–3] Oriental cultures have traditionally used plants rich in vegetable tannins to treat liver and kidney disease, arteriosclerosis, hypertension, and stomach ulcers. These tannins are also believed to play an important role in preventing tumor development and, in particular, tumors that are epithelial in nature. In humans, it is the oligomeric hydrolyzable tannins, *i.e.*, the ellagitannins and gallotannins, that appear to be the basis of reported medical activity.[4,5] These naturally occurring plant polyphenols have anti-mutagenic and anti-carcinogenic activities and appear to be universal inhibitors of the multistage pathways involved in carcinogenesis. While the precise mechanism(s) by which these compounds work is unclear, it has been suggested that their activity resides in their ability (1) to complex with and neutralize carcinogens, (2) to activate endogenous detoxification systems, and (3) to scavenge oxygen free radicals.[6–8]

Hydrolyzable tannins constitute a distinctive and unique group of plant metabolites. They are relatively large molecules (MW 20,000) and are thought to constitute one of the most important defense mechanisms of higher plants by providing a barrier against animal and microbial predators.[9] During digestion in humans, hydrolyzable tannins are degraded into gallotannins and ellagitannins. Several reports point to the anti-tumor activity of the gallotannins; however, the majority of evidence supports the role of ellagitannins in prevention of tumor development.[10] In the study reported here, attention has been focused on the ellagitannins and related compounds. Numerous experimental observations point to the potential role of hydrolyzable tannins and in particular one ellagitannin—ellagic acid—in preventing or inhibiting cancer.[11–14] Anti-carcinogenic effects of ellagic acid have been attributed to a variety of mechanisms, including the inhibition of metabolic activation of carcinogens, binding with the active forms of carcinogens, the protection

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of DNA, and stimulation of glutathione-*S*-transferase activity, resulting in activation of detoxification pathways.

Several food groups, including raspberries, strawberries, grapes, pecans, and walnuts, are rich in ellagitannins, but these vary in the relative amounts of the complex polyphenolic compounds. Phytochemicals such as ellagic acid may have anti-carcinogenic activity either alone or in association with other plant metabolites or host factors.

Development of cancer involves several molecular changes leading to the uncontrolled replication of a clone of transformed cells. The initiating lesion is a mutation, which affects the expression of oncogenes or tumor-suppressor genes or causes modification of their gene products. The berry extracts we have examined clearly contain substances that inhibit this first step in cancer formation. Initial experiments were performed with freshly pressed, unfractionated strawberry and raspberry juices. The activity observed with these fruits led us to begin a search for the anti-cancer substances in various small fruits by fractionation of fruits grown under defined conditions. Because of the complications that fats present in performing plant extractions, nuts were omitted from this study.

The assay used in this study is that developed by Ames *et al*.[15,16] This accurately identifies both direct-acting mutagens and those compounds that do not alter DNA directly, but are metabolically activated into mutagens by eukaryotic enzyme systems. In this assay, compounds are tested for the capacity to mutate strains of *Salmonella typhimurium* incapable of synthesizing the amino acid histidine (his^- auxotrophs) to the his^+ phenotype. For those compounds that are not direct-acting mutagens, liver microsomal extract is added to carry out the chemical transformations that occur *in vivo*. There is a 90% correlation between the mutagenicity as determined by this assay and carcinogenicity as determined by animal studies.[17] Styles[18] found that a close relationship exists between the mutagenic potency of a chemical in the Ames assay and its carcinogenic potency in animals.

MATERIALS AND METHODS

Mutagenesis assays

The mutagenicity assay protocol of Maron and Ames[19] was used with *S. typhimurium* strain TA100 as the test organism. This is a his^- base substitution mutant. Final dilutions of the extracts in the reaction mixture were such that the number of colonies on plates supplemented with extract (or extract plus S9 mix), but lacking mutagen, were the same as the number on plates containing solvent, but no extract. Methyl methanesulfonate (MMS; Sigma Chemical Co., St. Louis, MO) was used as the direct-acting mutagen. The indirect mutagen was benzo[*a*]pyrene dissolved in ethanol (BP; Sigma). This was activated by an S9 liver microsomal fraction prepared from Aroclor 1254-induced Sprague–Dawley rats (Bioreliance Corp., Rockville, MD). For MMS mutagenesis experiments the final dilution of extract in the reaction mixture was 1:12. For the BP experiments, this dilution was 1:27. For the MMS assays the top agar was composed of 2.0 mL of agar, 0.2 mL of bacterial culture, 0.2 mL of the berry extract to be tested (or its solvent), 0.2 mL of double-distilled water, and 1 μL of MMS freshly diluted 1:10 with water. For the BP mutagenesis experiments, 10% S9 mix was prepared as described by Maron and Ames.[19] This was divided into 0.5-mL aliquots, and 10.0 μL of BP was added to each. These were incubated at 37°C for 30 minutes and then added to tubes containing 2.0 mL of agar, 0.1 mL of bacterial culture, and 0.1 mL of berry extract. In controls, an equal volume of the appropriate solvent replaced the berry extract. In each assay plating was done in triplicate. Each assay was repeated three times as independent experiments. For each experiment, the mean number of colonies on the test plates was divided by the mean number on the control plates. These normalized values were averaged for the three experiments performed on a particular extract. Results are presented as the mean for the three experiments ($n = 9$). To assess the effect of a particular extract on mutagenesis, the mean of the normalized values for samples containing extract was divided by the mean value for samples containing mutagen alone. Values for this ratio smaller than 1.0 represent inhibition of mutagenesis by the extract.

To determine if antioxidants in the fruits contribute to the anti-mutagenic activity observed, a solution of ascorbic acid was prepared to obtain a pH equivalent to that of the strawberry juice (pH 3.6). This solution was tested in the same way as the fruit extracts.

Preliminary bioactivity screen

Strawberries and raspberries from a local market were washed thoroughly with tap water followed by double-distilled water and then homogenized in a blender. The juice was filtered through Whatman (Clifton, NJ) #1filter paper to remove particulate matter. It was then sterilized by filtration through a 0.20-μm-pore-size filter (Nalgene Labware, Rochester, NY). In one set of experiments, the pulp obtained by centrifugation was extracted by the addition of an equal volume of water. It was then sterilized as described above.

Bioassay-guided fractionation

The experimental approach regarded as most practical for natural product and cancer chemopreventive drug discovery is bioassay-directed fractionation.[20,21] As a first step toward successfully separating and identifying components with anti-mutagenic activity, extracts were prepared from specific varieties grown under commercial production. For this phase of the study, the strawberry cultivars Sweet Charlie and Carlsbad were obtained from BBI Produce, Dover, FL. Blueberries were obtained from the Small Fruit Research Station, Agricultural Research Service, U.S. Department of Agriculture, Poplarville, MS. In order to target separate classes of phytochemicals with potential activity we chose to perform a series of sequential targeted extractions to se-

lectively isolate polyphenols, flavanols, hydrolyzable tannins, ellagitannins, and other compounds.

Freeze-dried fruits of strawberry and blueberry were extracted twice sequentially in order of increasing polarity using hexane, 1:1 (vol/vol) hexane:ethyl acetate, ethyl acetate, and ethanol at room temperature. The extracts were filtered through Whatman #1 paper, and the solvents were evaporated at reduced pressure at 40°C. The ethyl acetate and ethanol fractions were tested in the mutagenesis assays.

In a second extraction process, strawberry fruits were extracted sequentially in reverse order of polarity in order to eliminate nuisance carbohydrates present in the previous extraction process. Frozen berries were first extracted with 7:3 (vol/vol) acetone:H_2O and evaporated under reduced pressure at 40°C to remove the acetone. The resulting solution was sequentially extracted with diethyl ether, ethyl acetate, and butanol. The butanol extract containing hydrolyzable tannins was evaluated in the mutagenesis studies. Because of the high level of anti-mutagenic activity observed for this extract, it was further fractionated with a lipophilic Sephadex LH-20 (Sigma-Aldrich, St. Louis) column using ethanol as the solvent. Fractions were collected as 300-mL aliquots, and similar fractions were combined according to their thin-layer chromatography profiles prior to bioassay. Eight fractions had antimutagenic activity.

RESULTS

Effects on mutagenesis

Effects of the berry extracts on MMS mutagenesis are summarized in Table 1. Their effects on mutagenesis by the metabolically activated carcinogen BP are summarized in Table 2. All of the extracts examined were effective in suppressing mutagenesis to some extent. For the unfractionated berry extracts addition to the plating agar resulted in a dilution of 1:13 in the MMS experiments and 1:27 in the BP experiments. Lyophilized extracts were diluted in 1.0 mL of solvent and then diluted in the same way.

Strawberries

Freshly squeezed, unfractionated juice from commercially obtained strawberries suppressed MMS mutagenesis by 37% and BP carcinogenesis by 76%. The hydrolyzable tannin-rich (butanol) extract from Sweet Charlie strawberries suppressed MMS mutagenesis by 65% and BP mutagenesis by 53%. The ethyl acetate extract containing flavanoid-glycosides and low-molecular-weight proanthocyanadins was much less active, inhibiting MMS mutagenesis by 22% and BP mutagenesis by 23%.

To better isolate hydrolyzable tannins, the extract of the Sweet Charlie strawberry was separated by Sephadex LH-20 gel filtration chromatography. The fractions were collected, the solvent was evaporated, and the residue was lyophilized and weighed. Since all of these fractions came from the same hydrolyzable tannin extract and were lyophilized immediately after collection, it was possible to compare their biological effectiveness directly. The fractions were assayed for their capacity to inhibit MMS-induced mutagenesis. A plot of percent inhibition versus fraction number is shown in Figure 1. All fractions were diluted as described in Materials and Methods. However, each fraction contained a different mass after lyophilization, resulting in different concentrations in the test agar. Here there has been no correction for differences in concentration. While some fractions had no effect on mutagenesis, others suppressed mutagenesis by over 50%. Data for the different fractions

TABLE 1. BERRY EXTRACT EFFECTS ON MUTAGENESIS BY MMS

Extract	*MMS*	*MMS + extract*	*Ratio*	*Inhibition (%)*
Commercial unknown strawberry				
Juice	14.81 ± 5.01	8.20 ± 1.43	0.63	37
Crude H_2O extract	16.96 ± 2.06	8.92 ± 1.36	0.53	47
Strawberry cultivar Sweet Charlie				
Hydrolyzable tannins extract	13.55 ± 0.82	4.82 ± 1.37	0.35	65
Flavanoid-glycosides and low-molecular-weight pro-anthocyanins	12.60 ± 1.54	9.72 ± 0.23	0.78	22
Anthocyanin-rich extract	8.45 ± 1.94	1.45 ± 0.82	0.17	83
Raspberry (commercial)				
Juice	12.77 ± 4.83	5.91 ± 2.64	0.43	57
Rabitteye blueberry cultivar Premier				
Ethanol anthocyanin-rich extract	19.67 ± 1.84	14.78 ± 0.43	0.76	24
Methanol extract	15.71 ± 1.53	10.22 ± 1.05	0.66	34
Rabitteye blueberry cultivar Tifblue				
Ethanol anthocyanin-rich extract	14.76 ± 2.46	7.87 ± 0.11	0.57	43
Ascorbic acid	10.26 ± 1.46	11.25 ± 1.54	1.10	0

Each extract was present in the final reaction mixture at a dilution of 1:13. Data are presented as ratios of the mean number of mutants for cells treated with mutagen plus extract to mean number of mutants for cells treated with mutagen alone. Error estimates represent standard error of the mean.

TABLE 2. BERRY EXTRACT EFFECTS ON MUTAGENESIS BY BP

Extract	*BP*	*BP + extract*	*Ratio*	*Inhibition (%)*
Commercial unknown strawberry				
Juice	3.42 ± 0.57	0.83 ± 0.12	0.24	76
Crude H_2O extract	3.35 ± 0.61	1.98 ± 0.29	0.63	37
Strawberry cultivar Sweet Charlie				
Hydrolyzable tannins extract	3.10 ± 0.31	1.46 ± 0.73	0.47	53
Flavanoid-glycosides and low-molecular-weight pro-anthocyanins	3.44 ± 0.65	2.57 ± 0.21	0.71	23
Raspberry (commercial)				
Juice	5.08 ± 0.65	2.66 ± 0.54	0.51	49
Rabitteye blueberry cultivar Premier				
Ethanol anthocyanin-rich extract	3.99 ± 0.80	2.08 ± 0.50	0.51	49
Methanol extract	3.84 ± 0.72	2.34 ± 0.44	0.61	39
Rabitteye blueberry cultivar Tifblue				
Ethanol anthocyanin-rich extract	3.78 ± 0.44	2.00 ± 0.50	0.52	48
Ascorbic acid	2.60 ± 0.19	2.69 ± 0.17	1.03	0

Each extract was present in the final reaction mixture at a dilution of 1:27. Data are presented as ratios of the mean number of mutants for cells treated with mutagen plus extract to mean number of mutants for cells treated with mutagen alone. Error estimates represent standard error of the mean.

were compared by calculating a specific antimutagenic activity, defined as:

Specific activity = % inhibition/extract concentration

where % inhibition = 1.0 – (number of mutants in the sample containing both extract and MMS divided by the number of mutants in the sample containing MMS only), and extract concentration represents the concentration (in mg/mL) of lyophilized extract in the top agar.

Specific activities for the various fractions are presented in Figure 2. Based on these results, a fraction containing anthocyanins from Sweet Charlie was isolated and proved highly effective as an inhibitor of MMS-induced mutagenesis, suppressing mutation induction by 83% (Table 1).

Other berries

Freshly squeezed, unfractionated raspberry juice from commercial raspberries suppressed MMS-induced mutagenesis by 57% and BP mutagenesis by 49% (Table 1). The alcohol extracts from blueberries exhibited suppression for both mutagens. Antioxidants inactivate free radical species, which can be mutagenic. As a result, these substances have been hypothesized to inhibit the development of cancer. As a control to determine whether antioxidants in the berry juices or their extracts contribute to the anti-mutagenic effects observed, a solution of ascorbic acid in water was prepared to have the same pH as that of strawberry juice (pH 3.6). This solution had no effect on the production of mutations by either the direct-acting carcinogen or by the one

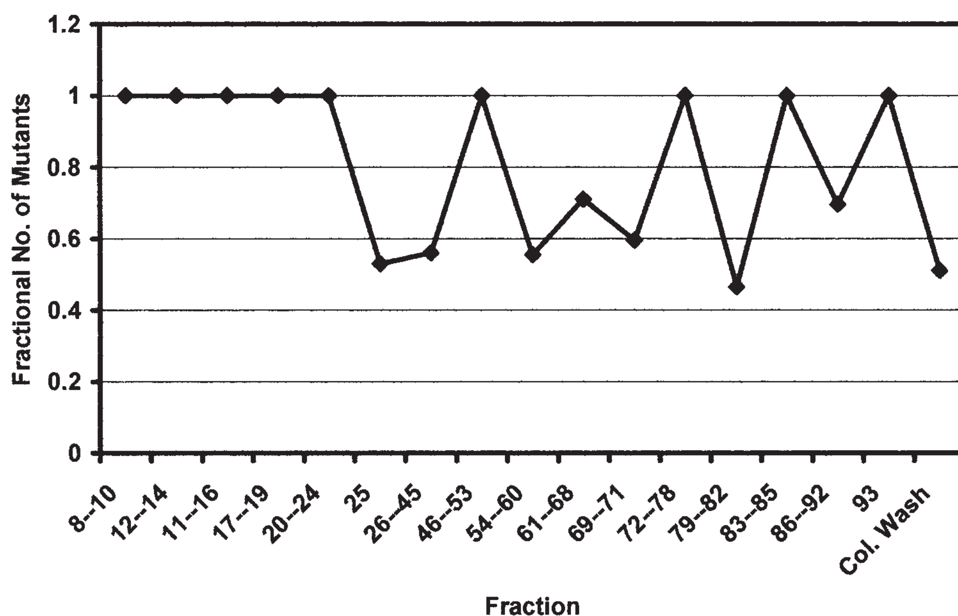

FIG. 1. Elution profile for the cultivar Sweet Charlie strawberry from Sephadex LH-20 gel filtration. Fractions were assayed for the ability to affect MMS-induced mutagenesis.

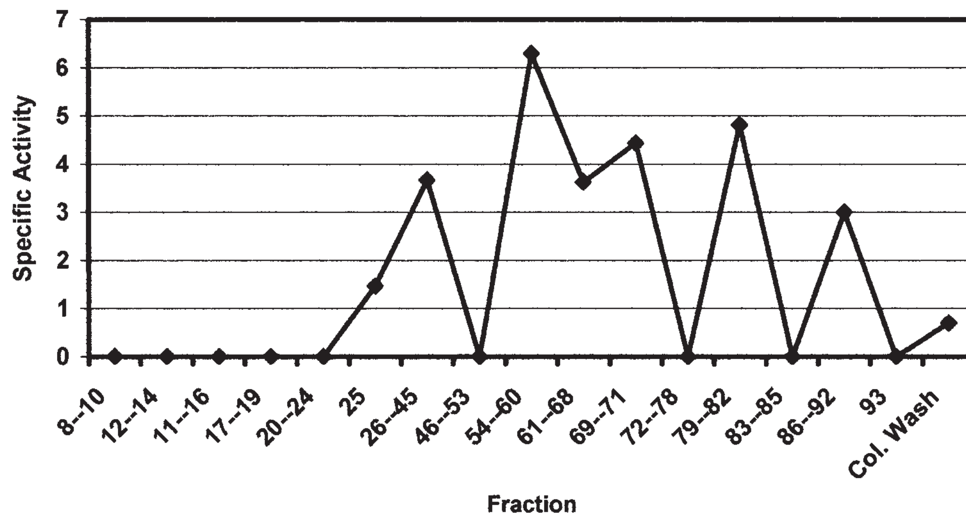

FIG. 2. Specific activities for the fractions shown in the elution profile of Figure 1. See Results for the definition of specific activity.

requiring metabolic activation. Bala and Grover[22] found a concentration-dependent anti-mutagenic effect of vitamin C. However, at the concentrations found in many fruits, no effect on mutagenesis could be detected.

DISCUSSION

Strawberries, raspberries and blueberries contain substances capable of protecting cells from mutation–the initiating event in carcinogenesis. This activity cannot be attributed (at least solely) to antioxidant activity. The anthocyanin-rich extract from strawberries was highly effective at inhibiting MMS mutagenesis, but the amount of this extract that could be obtained was insufficient to test its effect on BP mutagenesis. However, most of the extracts studied had similar effects in both assays.

These data are consistent with numerous reports that ellagitannins can be effective inhibitors of tumor initiation and promotion for several types of cancer in humans.[13,23–26] One such compound with demonstrated anti-cancer activity is ellagic acid. However, ellagic acid is only one of many phenolic compounds present in fruits, and the extent of bioactivity of these and their roles in tumor biology are still speculative. *In vivo* studies by Stoner and colleagues indicated that ellagic acid is active locally against esophageal carcinoma.[12,14] However, it is inactive in the rat mammary carcinogenesis model,[27] possibly because of poor absorption. Stoner[7] proposed that when humans consume fruits and nuts, the glucose moieties of ellagitannins are probably removed by enzymatic activity in the digestive system, thus "freeing up" ellagic acid for absorption. Numerous derivatives of ellagic acid exist in plants, formed through methylation, glycosylation, and methoxylation of its hydroxyl groups. These differ in solubility, mobility, and activity in plant as well as in animal systems.[6,11] The antioxidant activity of ellagic acid and its inhibition of cytochrome systems, which activate pro-carcinogens, probably both contribute to its tumor expression activity.[14] Both of these activities may be mediated by the quinone forms of ellagic acid. The dietary sources, digestibility, distribution, and organ accumulation of ellagitannins are, for the most part, unknown and need to be studied.

The search for and characterization of cancer-preventing compounds in fruits can have crucial roles in dietary prevention of this disease. Preliminary biological studies indicate that several of the extracts tested in this study also strongly inhibit metabolism of cancer cell lines.[28] Identification of the active components will allow breeding and genetic manipulation to enhance the amounts of beneficial phytochemicals in food products. The data reported here provide justification for further *in vivo* characterization of the chemopreventive compounds in berries. These data help clarify the anti-cancer benefits attributed to small fruits relevant to the initiation stage of carcinogenesis. However, clinical trials are essential in order to ultimately establish bioavailability and efficacy in humans.

Increasing evidence suggests that plant cellular defenses may be analogous to the "natural" immune response of vertebrates and insects.[5,29] In addition to cell structural similarities, plant and mammalian defense responses share functional similarities. Similar biosynthetic processes involved in signaling pathogen invasion and stress in plants and animals may account for the physiological cross activity of pharmacologically active phytochemicals such as resveratrol, ellagic acid, and various pentacyclic fatty acids.[30] Identification of individual compounds or classes of compounds that are important chemopreventative phytochemicals is an essential beginning to the development of functional foods and value-added crops. Ultimately, knowledge of the *in planta* role that pharmacologically active phytochemicals have and an understanding of elicitation and biometabolism will help facilitate the development of genetically enhanced cancer-fighting fruits and vegetables and better disease-resistant crops.

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