

# Berries

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## INTRODUCTION

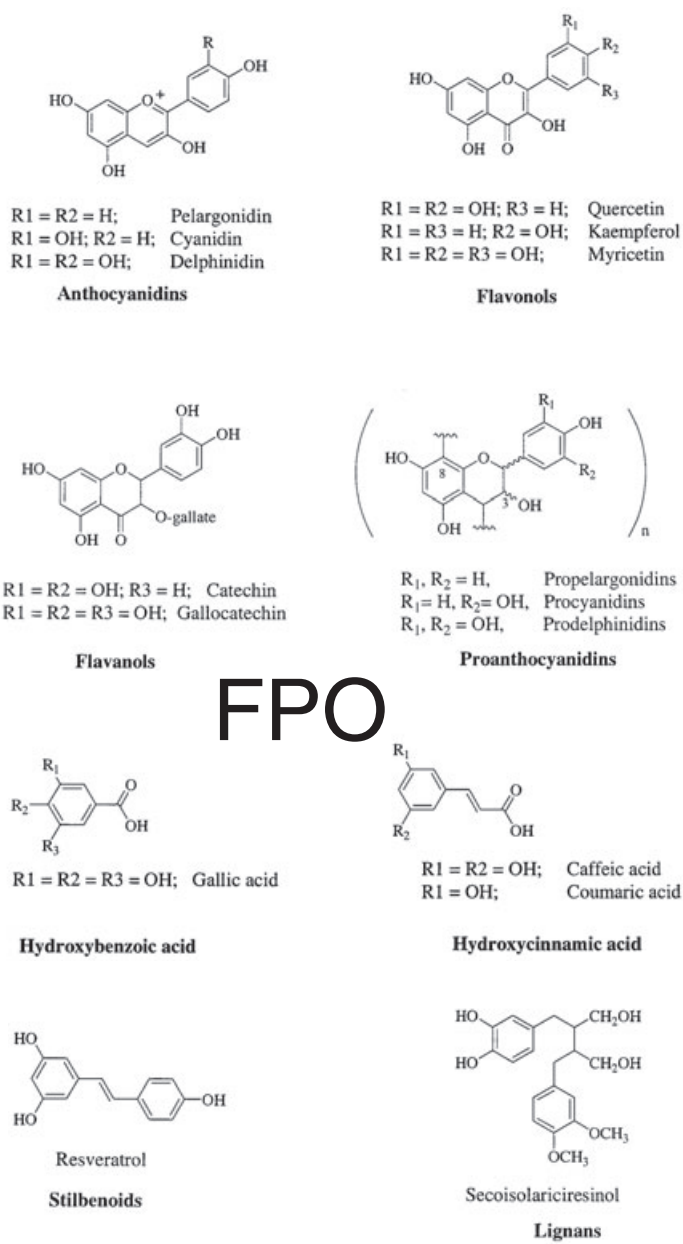
Studies suggest that consumption of a phytochemical-rich diet, which includes fruits and vegetables, contributes toward reducing the risk of certain types of human cancers. Among fruits, berries such as blackberries, black raspberries, blueberries, cranberries, raspberries, and strawberries are popularly consumed in our diet in fresh and in processed forms such as beverages, yogurts, jellies, and jams. In addition, berry extracts are widely consumed in dietary supplement forms for their potential human health benefits. A wide number of laboratory and animal studies have shown that berries have anticancer properties that are attributed to their high content of a diverse range of bioactive phytochemicals. These compounds include flavanoids (anthocyanins, flavonols, and flavanols), condensed tannins (proanthocyanidins [PAs]), hydrolyzable tannins (ellagitannins [ETs] and gallotannins [GTs]), stilbenoids (e.g., resveratrol), phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and lignans. Berry bioactives may impart anticancer effects through various complementary and overlapping mechanisms of action. These include antioxidant effects as free radical scavengers as well as acting indirectly through antioxidant actions that protect DNA from damage, the regulation of enzymes important in metabolizing xenobiotics and carcinogens, the modulation of nuclear receptors, gene expression, and subcellular signaling pathways of proliferation, angiogenesis, and apoptosis. This chapter reviews the progress, advances, future challenges, and impact of berry consumption on cancer prevention.

## STRUCTURAL TYPES OF BERRY BIOACTIVES

Over the past few decades, knowledge of the composition of berry fruits in fresh, freeze-dried, and extract forms has rapidly expanded with the advent of highly sensitive analytical methods, allowing researchers to establish phytochemical profiles or “chemical fingerprints” of these fruits. These bioactive phytochemicals have been identified as flavanoids (anthocyanins, flavonols, and flavanols), condensed tannins (PAs), hydrolyzable tannins (ETs and GTs), stilbenoids, phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and lignans (Seeram and Nair, 2002; Manach et al., 2004). The considerable diversity in the skeletal structures of these compounds imparts unique biological properties to each class that affects their absorption, distribution, metabolism, and excretion in humans (Beecher, 2003; Manach et al., 2004, 2005; Williamson and Manach, 2005). The structural diversity of berry bioactives can be observed by the varying types and oxidation levels of their heterocycle ring, their substitution patterns of hydroxylation (bearing an -OH group), the existence of stereoisomers, their glycosylation by various sugars, and/or acylation by organic and phenolic acids, and by conjugation with themselves to form polymers, etc. The main structural classes of berry bioactives are shown in Figure 1 and are discussed in the following subsections.

### Anthocyanins and Anthocyanidins

Of the numerous phytochemicals found in berries, the anthocyanins are probably the best known. Anthocyanins are



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FIGURE 1 Structures of bioactives found in berries: anthocyanins, flavonol, flavanols, proanthocyanidins, ellagitannins, hydroxycinnamic and hydroxybenzoic acids, stilbenoids, and lignans.

the pigments that impart the attractive red, blue, purple, violet, and intermediate red-purple to berries and many other fruits, vegetables, and grains (Mazza and Miniati, 1993; Seeram et al., 2001a,b; Wu and Prior, 2005). Similar to most other flavanoids, anthocyanins occur naturally in fruits and vegetables as glycosides. The de-glycosylated or aglycone forms of anthocyanins are known as *anthocyanidins*, which exist as different chemical structures, both colored and uncolored, according to variations in pH (Seeram et al., 2001b). Several hundred anthocyanins are known varying in the basic anthocyanidin skeleton, with the six most common being cyanidin (the most ubiquitous), delphinidin, pelargonidin, malvidin, petunidin, and peonidin (Seeram and Nair, 2002; Seeram et al., 2003) (Figure 1). Apart from variations in the anthocyanidin core, structural diversification is further achieved by the identity and extent and position at which glycosides and/or acyl groups are attached to the skeleton. The most common glycosides encountered on anthocyanins are glucose, galactose, rhamnose, and arabinose usually as 3-glycosides or as 3,5-diglycosides. However, rutinosides (6-*O*-*L*-rhamnosyl-*D*-glucosides), sophorosides (2-*O*-*D*-glucosyl-*D*-glucosides), and sambubiosides (2-*O*-*D*-xylosyl-*D*-glucosides) are also common, as are some 3,7-diglycosides and 3-triosides (Clifford, 2000). Common acylating agents include phenolic acids (caffeic, *p*-coumaric, ferulic, and sinapic), which may themselves bear glycosidic sugars, and a range of aliphatic acids (e.g., acetic, malic, malonic, oxalic, and succinic).

### Flavonols

Flavonols are the most ubiquitous flavonoids found in foods and similar to anthocyanins also occur naturally in glycosylated forms where the associated sugar moiety is very often glucose or rhamnose, although other substituents such as galactose, arabinose, xylose, and glucuronic acid may also be involved. The most common flavonol aglycons are quercetin (the most ubiquitous), kaempferol, and myricetin (Figure 1). Because their biosynthesis is stimulated by light, flavonols accumulate in the outer and aerial tissues of plants (in leaves) and fruits (in skin). However, in berries, they are commonly found in the flesh and achenes of the fruits such as in strawberries (Seeram et al., 2005) and in raspberries (Kaehkoenen et al., 2001).

### Flavanols

Flavanols are the only class of flavonoids that do not occur naturally as glycosides. Flavanols exist in both monomeric (catechins) and polymeric (PAs; see later discussion) forms (Figure 1). Catechins and epicatechin are the main flavanols found in berry fruits, whereas gallicocatechin, epigallocatechin, and epigallocatechin gallate are found in certain seeds of leguminous plants, in grapes, and in tea (Manach et al., 2004).

### Condensed Tannins (Proanthocyanidins)

Condensed tannins, or PAs, are dimers, oligomers, and polymers of catechins that are bound together by links between C4 and C8 (or C6) (Buelga-Santos and Scalbert, 2000) (Figure 1). However, in addition to C–C linkages, PAs can also have ether linkages between C2–O5 or C2–O7, referred to as *A-type linkages*. PAs can also be classified based on their constituent units that are produced on acid hydrolysis. In this case, they are referred to by the nomenclature system established for anthocyanidins. The most ubiquitous PAs are procyanidins, consisting of individual (epi)catechin units. Others include the less common pro-pelargonidins, consisting of (epi)afzelechin units, and prodelphinidins, consisting of (epi)gallicocatechin units. It is difficult to estimate the PA content of berries and other foods because they have a wide range of structures and molecular weights and their mean degree of polymerization in foods has rarely been determined (Manach et al., 2004; Gu et al., 2004). Among common edible berries, blueberries and cranberries contain high levels of PAs (Gu et al., 2004).

### Hydrolyzable Tannins (Ellagitannins and Gallotannins)

Hydrolyzable tannins are categorized into GTs and ETs. GTs are esters of gallic acid, whereas ETs are composed of esters of hexahydroxydiphenic acid (HHDP: 6,6'-dicarbonyl-2,2',3,3',4,4'-hexahydroxybiphenyl moiety). On hydrolysis of ETs, the HHDP moiety spontaneously rearranges to release ellagic acid, hence, their name (Clifford and Scalbert, 2000). The hydrolysis reaction to form ellagic acid is usually employed for the detection and quantification of ETs in berry fruits (Amakura et al., 2000). ET monomers can be further oxidized in plants and form dimers, trimers, and tetramers with molecular weights up to 4000 Da. Among common edible berries, strawberries, raspberries, blackberries, and black raspberries contain high levels of ETs (Maeettae-Riihinen et al., 2004a,b).

### Phenolic Acids

Berries contain a wide variety of phenolic acids (Zadernowski et al., 2005), which occur as derivatives of hydroxybenzoic acid (e.g., gallic acid) and hydroxycinnamic acid (e.g., caffeic acid) (Figure 1). The hydroxycinnamic acids are more common than the hydroxybenzoic acids and consist chiefly of *p*-coumaric, caffeic, ferulic, and sinapic acids. Hydroxycinnamic acids are found in all parts of fruits, although the highest concentrations are seen in the outer parts of ripe fruits. Concentrations generally decrease during the course of ripening, but total quantities increase as the fruit increases in size (Clifford, 2004). Although phenolic acids are found in berries in free forms, they can be considered components of complex polymers such as hydrolyzable

tannins (GTs or ETs) or PAs. Hence, they can be released from their “parent” compounds *in vivo* by changes in physiological pH, enzymatic, or gut bacterial action, or *in vitro* by storage, processing conditions, and so on (see the section “Bioavailability and Metabolism of Berry Bioactives”).

### Stilbenoids

Stilbenes are phenolic-based compounds, of which the most widely recognized is resveratrol (3,4',5-trihydroxystilbene) (Figure 1). Resveratrol has attracted immense attention because of its biological properties including its anticancer effects (reviewed in Aggarwal et al., 2004). Resveratrol has a number of closely related analogs (e.g., pterostilbene and piceatannol), and it plays an important role as the parent molecule of oligomers known as the *viniferins* (Aggarwal et al., 2004). Among edible berries, resveratrol and its analogs have been reported in members of the *Vaccinium* genus, for example, blueberry, bilberry, lingonberry, and cranberry (Rimando et al., 2004).

### Lignans

Lignans are formed from two phenylpropane units. Although the richest dietary sources of lignans are flaxseed and linseed, which contain secoisolariciresinol (Figure 1) and low quantities of matairesinol (Scalbert and Williamson, 2000; Clifford, 2004), some cereals, grains, fruits, and certain vegetables also contain traces of lignans. Lignans have been reported in berries such as strawberry, blackberry, raspberry, cloudberry, cranberry, lingonberry, and blueberry (Mazur et al., 2000).

### Triterpenes and Sterols

Although the predominant bioactive phytochemicals in berries are phenolic compounds (see previous subsections), there have been reports of nonpolar compounds such as ursolic acid, triterpene hydroxycinnamates, and  $\beta$ -sitosterol in members of the *Vaccinium* species, for example, cranberries (Murphy et al., 2003; Schmandke, 2004).

## DISTRIBUTION OF BERRY BIOACTIVES

Berries contain a wide range of phytochemicals, the most predominant of which are phenolic (aromatic ring bearing hydroxyl, -OH, group) in nature. Phenolic contents have been reported to vary considerably among different berry genera. For example, in the *Vaccinium* genus, anthocyanins are reported as the main phenolics in bilberry, bog-whortleberry, and cranberry, but in cowberries, flavanols, and procyanidins predominate (Kaehkoenen et al., 2001). In the

genus *Rubus* (e.g., cloudberry and raspberry), the main phenolics are ETs, and in the genus *Fragaria* (e.g., strawberry), ETs are the second largest group after anthocyanins (Kaehkoenen et al., 2001). Flavonols based on quercetin and kaempferol aglycons are also reported to be present in substantial quantities in strawberries (Seeram et al., 2005). Phenolic acids are found in high levels in rowanberries (genus *Sorbus*) and anthocyanins in chokeberries (genus *Aronia*). In the genus *Ribes* (currants and gooseberries), anthocyanins predominate, as well as in crowberries (genus *Empetrum*) (Kaehkoenen et al., 2001).

Maeaettae-Riihinen (2004) identified and quantified soluble and insoluble phenolics in 18 species of berries belonging to the families Grossulariaceae, Ericaceae, Rosaceae, Empetraceae, Elaeagnaceae, and Caprifoliaceae. The berry phenolics were identified as conjugated hydroxycinnamic acids, flavonol glycosides, and anthocyanins. The study showed similarities in the distribution of conjugated forms of phenolics among berry species of the same family and differences in the profiles and compositions of anthocyanins among individual types of berries.

### Mechanisms of Chemoprevention by Berry Bioactives

Epidemiological evidence has shown that the consumption of a phytochemical-rich diet contributes toward reducing the risk of certain types of human cancers (Steinmetz and Potter, 1991; Meyskens and Zabo, 2005). Although the predominant phytochemical in berry fruits are phenolic compounds such as anthocyanins, its other phytochemicals may also contribute synergistically and/or additively to its anticancer activities (Camire, 2002; Seeram et al., 2004). Hence, berries contain a wide range of phytochemicals that may impart anticancer effects through various complementary and overlapping mechanisms of action (Liu, 2003; Heber, 2004). The individual constituents of berries, as well as total berry extracts, have been shown in *in vitro* and *in vivo* studies to exert anticancer properties through different mechanisms. For example, berry bioactives may exert anticancer effects through their antioxidant properties as free radical scavengers while acting indirectly through antioxidant actions that protect DNA from damage, the regulation of enzymes important in metabolizing xenobiotics and carcinogens, the modulation of nuclear receptors, gene expression, and subcellular signaling pathways of proliferation, angiogenesis, and apoptosis.

Although an effort has been made to categorize the actions of berry bioactives in the following discussion, the reader will notice overlap between sections, which is due to the multimechanistic and complementary pathways through which berry phytochemicals exert their anticancer effects.

### Modulation of Signaling Pathways of Proliferation, Apoptosis, and Cell Cycle Arrest

Studies have investigated the subcellular signaling and molecular mechanisms through which berry phytochemicals may exert their anticancer properties. These include the ability of berry extracts and their purified bioactives to inhibit cell proliferation and modulate cell cycle arrest, induce DNA repair and signal transduction, and apoptosis in cancer cells while having little or no cytotoxic effect on normal noncancerous cells. Although many of these studies have focused on evaluating whole berry extracts, a significant number of bioassay-guided fractionations, aimed at isolating and identifying the active constituents present in the berry extracts, have been done. Some of the *in vitro* anticancer studies conducted on berries are discussed in the following paragraphs.

Blueberries, black chokeberries, lingonberries, and raspberries extracts were shown to decrease the proliferation of human colon HT-29 and breast MCF-7 cancer cells in a dose-dependent manner (Olsson et al., 2004). Similarly, whole cranberry fruit extracts were assayed for tumor growth inhibition using seven tumor cell lines and selective inhibition of K562 leukemia, and HT-29 colon cells were observed from a methanolic extract in the range of 16–125  $\mu\text{g/ml}$  (Yan et al., 2002). Bilberry extract was shown to inhibit the growth of human HL60 leukemia cells and HCT116 colon carcinoma cells and induce apoptotic cell bodies and nucleosomal DNA fragmentation in the HL60 leukemia cells (Katsube et al., 2003). Juranic (2005) compared the antiproliferative action of red raspberries to malignant human colon carcinoma LS174 cells and to normal immune competent cells, with the action of ellagic acid (a bioactive constituent of berries) alone. Results from this study showed that the raspberry extracts possess the potential for antiproliferative action against human colon carcinoma cells, which was correlated with its content of ellagic acid. In this study, the cytotoxic activity of the extracts was not pronounced on normal human PBMC colon cells (Juranic, 2005).

A bioactivity-guided fractionation of cranberries identified triterpenoid esters that inhibited the growth of MCF-7 breast, ME180 cervical, and PC3 prostate tumor cell lines (Murphy et al., 2003). The major bioactives were identified as the *cis*- and *trans*- isomers of 3-*O*-*p*-hydroxycinnamoyl ursolic acid. The authors reported that the *cis*- isomer showed superior antiproliferative activity when compared to its *trans* counterpart and to quercetin and cyanidin-3-galactoside. Phenylboronic acid was also isolated from the cranberry extract, but it did not exhibit significant antitumor activity (Murphy et al., 2003).

Ramos (2005) investigated the effects of individual purified berry bioactives, quercetin, chlorogenic acid, and

epicatechin, as well as whole strawberry fruit extract on the viability and apoptosis of human hepatoma HepG2 cells. Quercetin and the strawberry fruit extract inhibited cell viability in a dose-dependent manner, whereas chlorogenic acid and epicatechin had no prominent effects on the cell death rate. Similarly, quercetin and the strawberry extract, but not chlorogenic acid and epicatechin, induced apoptosis in hepatoma HepG2 cells. In cell cycle progression experiments, quercetin and the strawberry extract were observed to arrest the G<sub>1</sub> phase in the cell cycle before apoptosis (Ramos et al., 2005).

In a study to identify the chemopreventive phytochemicals in black raspberries, Han et al. (2005) identified two of its bioactives as ferulic acid and  $\beta$ -sitosterol. In the bioassay-guided fractionation experiment, Han (2005) also demonstrated that a purified fraction eluted with ethanol during chromatography of the organic extract of freeze-dried black raspberries inhibited the growth of premalignant and malignant but not normal human oral epithelial cell lines. However, purified ellagic acid alone was found to inhibit the growth of normal, premalignant, and malignant human oral cell lines. Using flow cytometry and Western blotting of cell cycle-regulatory proteins, these workers also investigated molecular mechanisms by which ferulic acid,  $\beta$ -sitosterol, and the berry ethanol fraction could selectively inhibit the growth of premalignant and malignant oral cells. They observed no discernible change in the cell cycle distribution following treatment of cells with the berry ethanol fraction. Premalignant and malignant cells redistributed to the G<sub>2</sub>/M phase of the cell cycle following incubation with ferulic acid, whereas  $\beta$ -sitosterol treated premalignant and malignant cells accumulated in the G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M phases. The berry ethanol fraction reduced the levels of cyclin A and cell division cycle gene 2 (*cdc2*) in premalignant cells and cyclin B1, cyclin D1, and *cdc2* in the malignant cell lines. The berry ethanol fraction also elevated the levels of p21<sup>waf1/cip1</sup> in the malignant cell line. Ferulic acid treatment led to increased levels of cyclin B1 and *cdc2* in both cell lines, and p21<sup>waf1/cip1</sup> was induced in the malignant cell line; on the other hand,  $\beta$ -sitosterol reduced the levels of cyclin B1 and *cdc2* while increasing p21<sup>waf1/cip1</sup> in both the premalignant and the malignant cell lines. The authors concluded that the growth-inhibitory effects of black raspberries on premalignant and malignant human oral cells may reside in specific components that target aberrant signaling pathways regulating cell cycle progression (Han et al., 2005).

Cell lines of differing origins have been shown to respond with varying degrees of sensitivity in growth toward berry extracts. For example, a cranberry presscake (the material remaining after squeezing the juice from berries) extract inhibited proliferation of eight human tumor cell lines of multiple origins (Ferguson et al., 2004). The androgen-dependent prostate cell line, LNCaP, was the most sensitive

of those tested, whereas the estrogen-independent breast line, MDA-MB-435, and the androgen-independent prostate line, DU145, were the least sensitive. Other human tumor lines originating from breast (MCF-7), skin (SK-MEL-5), colon (HT-29), lung (DMS114), and brain (U87) had intermediate sensitivities to the cranberry extract. Using flow cytometric analyses of DNA distribution (for cell cycle) and annexin V positivity (for apoptosis), the authors showed that a purified fraction blocked cell cycle progression and induced cells to undergo apoptosis in a dose-dependent manner in MDA-MB-435 breast cells (Ferguson et al., 2004). In another study, human oral, prostate, and colon cancer cells responded with differing sensitivities to cranberry bioactives (Seeram et al., 2004). In this study, Seeram et al. (2004) showed that although the individual cranberry phytochemicals, such as its flavonols, organic acids, PAs, and anthocyanins, inhibited the growth of the tumor cells, when they were combined, as found in a total cranberry extract, antiproliferative activities were significantly enhanced.

The effects of berry extracts on signal transduction pathways have also been reported. For example, Huang (2002) reported on the effects of a black raspberry methanol extract and its purified fractions on transactivation of activated protein-1 (AP-1) and nuclear factor- $\kappa$ B (NF $\kappa$ B) induced by the carcinogen, BaP diol-epoxide (BPDE), in mouse epidermal cells. AP-1 and NF $\kappa$ B are transcription factors associated with carcinogenesis (Bode and Dong, 2000). Inhibition of AP-1 activity has been shown to lead to the suppression of cell transformation (Dong et al., 1997). NF $\kappa$ B is also an important regulator in deciding cell fate, such as programmed cell death and proliferation control, and is critical in tumorigenesis (Baldwin, 1996). The inhibitory effects of a purified black raspberry fraction on AP-1 and NF $\kappa$ B were mediated via inhibition of mitogen-activated protein kinase (MAPK) activation and inhibitory subunit  $\kappa$ B phosphorylation, respectively. Pretreatment of cells with purified berry fractions did not result in an inhibition of BPDE binding to DNA, which suggested that this was not a mechanism of reduced AP-1 and NF $\kappa$ B activities. In addition, none of the purified fractions were found to affect p53-dependent transcription activity. In view of the important roles of AP-1 and NF $\kappa$ B in tumor promotion and progression, the authors concluded that the ability of black raspberries to inhibit tumor development may be mediated by impairing signal transduction pathways, leading to activation of AP-1 and NF $\kappa$ B (Huang et al., 2002).

The inhibitory effects of strawberry on tetradecanoylphorbol-13-acetate (TPA)—or ultraviolet B (UVB)—induced AP-1 and NF $\kappa$ B were recently demonstrated by Wang (2005). TPA and UVB are well-known tumor promoters and can produce reactive oxygen species (ROS) and stimulate AP-1 and NF $\kappa$ B activities by activating MAPK signaling pathways such as the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino-terminal

kinases (JNKs), and the p38 MAPK (Schulze-Osthoff et al., 1997; Hou et al., 2004). These workers also evaluated strawberry extracts for inhibition of proliferation and transformation of human and mouse cancer cells. The strawberry extracts inhibited the proliferation of human lung epithelial cancer cell line A549 and decreased TPA-induced neoplastic transformation of mouse epidermal cells. In addition, pretreatment of the mouse epidermal cells with strawberry extracts resulted in the inhibition of both UVB- and TPA-induced AP-1 and NF $\kappa$ B transactivation. Furthermore, the strawberry extracts also blocked TPA-induced phosphorylation of ERKs and UVB-induced phosphorylation of ERKs and JNK kinase in the mouse epidermal cell culture. These results suggest that the ability of strawberries to block UVB- and TPA-induced AP-1 and NF $\kappa$ B activation might be due to their antioxidant properties and their ability to reduce oxidative stress. The authors concluded that the oxidative events that regulate AP-1 and NF $\kappa$ B transactivation could be important molecular targets for cancer prevention. Therefore, strawberries may be highly effective as chemopreventive agents that act by targeting the downregulation of AP-1 and NF $\kappa$ B activities, blocking MAPK signaling, and suppressing cancer cell proliferation and transformation (Wang et al., 2005).

In another study, freeze-dried strawberries and freeze-dried black raspberries were extracted with methanol, partitioned, and chromatographed into several fractions (Xue et al., 2001). The extracts, along with ellagic acid, were analyzed for anti-transformation activity in a Syrian hamster embryo (SHE) cell transformation model. None of the extracts or ellagic acid alone produced an increase in morphological transformation. For assessment of chemopreventive activity, SHE cells were treated with test samples and benzopyrene for 7 days. Ellagic acid and two of the purified fractions produced a dose-dependent decrease in transformation compared with the benzopyrene treatment only. Ellagic acid and the two purified fractions were further examined using a 24-hour co-treatment with benzopyrene or a 6-day treatment following 24 hours with benzopyrene. Ellagic acid showed inhibitory ability in both protocols. The two purified fractions significantly reduced benzopyrene-induced transformation when co-treated with benzopyrene for 24 hours. The authors concluded that the possible mechanism by which the purified fractions inhibited cell transformation appear to involve interference of uptake, activation, detoxification of benzopyrene, and/or intervention of DNA binding and DNA repair (Xue et al., 2001).

### Angiogenesis

Antiangiogenic (the ability to reduce unwanted growth of blood vessels) approaches to treat cancer represent a priority area in vascular tumor biology. 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.

Extracts of blueberry, bilberry, cranberry, elderberry, raspberry, and strawberry were studied for antioxidant efficacy, cytotoxic potential, cellular uptake, and antiangiogenic properties (Roy et al., 2002; Bagchi et al., 2004). The authors evaluated various combinations of the extracts and showed that a "synergistic extract" significantly inhibited both hydrogen peroxide ( $H_2O_2$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-induced vascular endothelial growth factor (VEGF, a key regulator of tumor angiogenesis) expression by human keratinocytes (Roy et al., 2002; Bagchi et al., 2004). The same research group also studied the synergistic extract in an *in vivo* mice model of angiogenesis and observed that it significantly inhibited basal monocyte chemotactic protein-1 (MCP-1), a protein responsible for facilitating angiogenesis (Atalay et al., 2003). In addition, the synergistic extract significantly inhibited inducible NF $\kappa$ B transcription. Endothelioma cells pretreated with the synergistic berry extract showed a diminished ability to form hemangioma and markedly decreased tumor growth by >50% (Atalay et al., 2003).

Liu (2005) reported that black raspberry extract showed antiangiogenic properties in a human tissue-based *in vitro* fibrin clot angiogenesis assay. Bioassay-guided fractionation of the berry extract resulted in a highly potent antiangiogenic fraction that completely inhibited angiogenic initiation and vessel growth. Further subfractionation of this active fraction revealed the coexistence of multiple antiangiogenic compounds, one of which has been identified as gallic acid (a bioactive constituent of berries). However, the authors concluded that the whole fraction was superior than its subfractions and that the active ingredients may be additive and/or synergistic in their antiangiogenic effects (Liu et al., 2005).

### Antimutagenicity

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. Strawberry, blueberry, and raspberry juices and extracts were evaluated for their ability to inhibit the production of mutations by the direct-acting mutagen methyl methanesulfonate and the metabolically activated carcinogen benzopyrene (Hope et al., 2004). The berry juices significantly inhibited mutagenesis caused by both carcinogens. Ethanol extracts from freeze-dried fruits of strawberry and blueberry cultivars were also evaluated, and hydrolyzable tannin-containing fractions from strawberries were found to be most effective at inhibiting mutations.

### Induction of Antioxidant Enzymes

ROS are formed during normal endogenous metabolic process and from exogenous factors such as ionizing radi-

tion, diet, and xenobiotics (Davis, 1987; Halliwell and Gutteridge, 1989). Oxidative stress arises either from the overproduction of ROS or from the deficiency of antioxidant defense or repair mechanisms, resulting in reversible or irreversible damage to critical cellular macromolecules such as lipids, proteins, and DNA (Davis, 1987). Oxidative stress has been implicated in initiation, promotion, and progression phases of carcinogenesis (Cerutti, 1985) and the resulting unrepaired oxidative damage has been suggested to play a role in other chronic diseases, including cancer (Ames et al., 1993). Aerobic organisms constantly battle the adverse effects of ROS by increasing the production of biochemical antioxidants (such as glutathione and ascorbate) or by inducing endogenous antioxidant enzymes including superoxide dismutase (SOD), catalase, glutathione peroxidase (G-POD), and glutathione reductase (GR). These scavenging antioxidant molecules and the endogenous antioxidant enzymes attenuate the ROS concentration to maintain an intracellular reduction and oxidation (redox) balance.

Strawberries have been shown to have antioxidant capacity against ROS such as ROO,  $O_2^-$ ,  $H_2O_2$ , OH, and  $^1O_2$  (Wang and Lin, 2000; Wang and Zheng, 2001). These workers also demonstrated the activities of antioxidant enzymes including SOD, G-POD, and GR in strawberries (Wang et al., 2005). The activities of antioxidant enzymes in blackberries have also been shown (Jiao and Wang, 2000). The activities of antioxidant enzymes in both berries were shown to be positively correlated to their antioxidant capacity (Jiao and Wang, 2000; Wang et al., 2005).

Studies have been designed to investigate correlations between antioxidative potential and antiproliferative activities of berries. For example, Meyers (2003) investigated eight strawberry cultivars to find out if their antioxidant capacities, by the total oxyradical scavenging capacity (TOSC) assay, can be correlated to their antiproliferative activities. Overall, although the proliferation of HepG2 human liver cancer cells was significantly inhibited in a dose-dependent manner after exposure to all strawberry cultivar extracts, these workers found no relationship between antiproliferative activity and antioxidant content. In another study, similar results were observed when extracts of four raspberry cultivars were evaluated for total antioxidant capacity and cancer cell antiproliferative activity (Weber and Liu, 2002). In this study, the antioxidant activity of each cultivar was directly related to the total amount of phenolics, but no significant relationship was found between antiproliferative activity and the total amount of phenolics (Weber and Liu, 2002).

Berry extracts and their purified bioactives have been investigated for effects on the production of cytokines such as TNF- $\alpha$ , which mediates a variety of cell functions including stimulation of nitric oxide (NO) production. TNF- $\alpha$  has been related to oxidative stress and diseases such as chronic inflammation (Park et al., 2000). Wang (2002) investigated common purified berry phenolics and anthocyanin-enriched

blueberry, blackberry, Saskatoon berries, and black currant extracts for their effects on the production of TNF- $\alpha$  in RAW 264.7 macrophages. Gallic acid and catechin showed small but significant effects, whereas chlorogenic acid had no effect on TNF- $\alpha$  production. The flavonol quercetin inhibited TNF- $\alpha$  production, whereas kaempferol and myricetin induced the secretion of TNF- $\alpha$ . The individual anthocyanidins (pelargonidin, cyanidin, delphinidin, peonidin, and malvidin), anthocyanins (malvidin 3-glucoside and malvidin 3,5-diglucosides), and anthocyanin-enriched blueberry extracts induced TNF- $\alpha$  production and acted as modulators of the immune response in the activated macrophages (Wang and Mazza, 2002).

### Inhibitors of Detoxification Enzymes

Phase I and phase II metabolizing enzymes play an important role in the biotransformation of carcinogens and xenobiotics in the human body. In phase I reactions, these chemicals undergo bioactivation catalyzed by cytochrome P450 (CYP) isozymes to produce strong electrophiles capable of interacting with cellular nucleophiles such as DNA to form adducts eventually culminating in mutagenesis and neoplastic transformation. In normal cells, the reactive intermediates formed by phase I reactions are then conjugated via phase II enzymes with glucuronides, sulfate, or glutathione, facilitating their excretion. Phase II xenobiotic detoxification enzymes include glutathione *S*-transferase, sulfotransferases, UDP-glucuronyl transferases, and quinone reductase (QR). Conjugation enhances hydrophilicity of the metabolites, thus facilitating elimination of the carcinogen from the body. While phase I enzymes increase the carcinogenic potency of a chemical, phase II enzymes serve to detoxify the electrophilic metabolites. An imbalance in phase I and phase II carcinogen-metabolizing enzymes has been documented in a wide range of malignant tumors including breast cancer (Williams and Phillips, 2000).

Kansanen et al. (1996) investigated the *in vitro* effects of some flavanoids and phenolic acids common to berries, as well as extracts of strawberry and black currant, on CYP 1A1 isozyme. These workers found that the flavonoid aglycons and berry extracts were effective inhibitors of CYP 1A1, whereas the flavonoid glycosides and phenolic acids were not (Kansanen et al., 1996). Other studies have shown that flavonoid-rich fractions from *Vaccinium* species, such as cranberries, induce QR *in vitro* (Bomser et al., 1996). This study also showed that a cranberry extract inhibited expression of ornithine decarboxylase (ODC), a key enzyme responsible for polyamine biosynthesis (Bomser et al., 1996).

### Inhibitors of Metalloproteinase Enzymes

Matrix metalloproteinases (MMPs) are enzymes essential for development and remodeling of tissues and aberrant

overexpression of these enzymes contributes to several pathological conditions. In particular, MMP overexpression in cancer plays a significant role in metastasis by providing a mechanism for invasion and progression. MMPs are involved in proteolysis of the extracellular matrix, which can lead to the progression of tumors (Pupa et al., 2002). Raspberries and blackberries have been shown to inhibit the activities of MMP-2 and MMP-9 (Tate et al., 2004). Quercetin, a typical berry flavonol, has been shown to have a chemoprotective role through complex effects on signal transduction involved in cell proliferation, including increased expression of endogenous tissue inhibitors of MMPs (Morrow et al., 2001). Ursolic acid, which has been reported in berries such as cranberries (Murphy et al., 2003), reduced the expression of MMP-9 in HT-1080 fibrosarcoma cells and consequently inhibited tumor invasion (Cha et al., 1996, 1998).

As part of a study to determine the effects of cranberry extracts on prostate tumor proliferation, evaluation of the effects of whole cranberry extract and purified fractions on MMP expression in DU145 prostate cells was conducted (Kondo et al., 2004; Neto et al., 2005). The whole cranberry extract inhibited expression of MMP-2 and MMP-9 in the cells at 100  $\mu$ g/ml concentrations. A purified PA fraction, at 500  $\mu$ g/ml, inhibited MMP-2 expression completely and resulted in ~75% inhibition of MMP-9 activity (Kondo et al., 2004; Neto et al., 2005).

## CHEMOPREVENTION STUDIES WITH BERRY BIOACTIVES

### Animal Studies

The chemopreventive potential of freeze-dried berries against aerodigestive tract cancers, such as oral cavity and esophageal cancers, has been demonstrated in a number of animal studies (Stoner et al., 1999; Kresty et al., 2001; Aziz et al., 2002; Casto et al., 2002). Studies have suggested that berries, which contain high amounts of ETs and ellagic acid (e.g., strawberries and black raspberries), show better effects against these cancers than those that contain PAs as their predominant tannins (e.g., blueberries) (Aziz et al., 2002). However, although ellagic acid, an abundant component in berries, has been shown to inhibit carcinogenesis both *in vitro* and *in vivo*, several studies have reported that other compounds in berries may also contribute to the observed anticancer effects (Stoner et al., 1999).

Studies have been designed to investigate the chemopreventive effects of berries during initiation and progression phases of cancer. Lyophilized black raspberries (LBRs) were evaluated against N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in the F344 rat during initiation and post-initiation phases of carcinogenesis

(Kresty et al., 2001). Anti-initiation studies included a 30-week tumorigenicity period, quantification of DNA adducts, and NMBA metabolism study. Feeding 5 and 10% LBRs, for 2 weeks prior to NMBA treatment and throughout a 30-week period significantly reduced tumor multiplicity (39 and 49%, respectively). The post-initiation inhibitory potential of berries was evaluated in a second experiment with administration of LBRs after NMBA treatment. In this experiment, animals were sacrificed at 15, 25, and 35 weeks. The LBRs were found to inhibit tumor progression as evidenced by significant reductions in the formation of preneoplastic esophageal lesions, decreased tumor incidence and multiplicity, and reduced cellular proliferation. At 25 weeks, both 5 and 10% LBRs significantly reduced tumor incidence, tumor multiplicity, proliferation rates, and preneoplastic lesion development. At 35 weeks, only the 5% LBRs significantly reduced tumor incidence and multiplicity, proliferation indices, and preneoplastic lesion formation. The authors concluded that dietary administration of LBRs inhibited events associated with the initiation, promotion, and progression stages of carcinogenesis.

In another study with LBRs, the hamster cheek pouch (HCP) assay was used to evaluate the ability of the berries to inhibit oral cavity tumors (Casto et al., 2002). Hamsters were fed 5% and 10% LBRs in the diet for 2 weeks prior to treatment with dimethylbenzanthracene (DMBA) and for 10 weeks thereafter. HCPs were painted with the DMBA to induce tumor formation. The animals were sacrificed 12–13 weeks after the beginning of DMBA treatment and the number and volume of tumors were determined. The authors observed a significant difference in tumor number between the LBR-treated and control groups (Casto et al., 2002).

Blueberries were evaluated for their ability to inhibit NMBA tumorigenesis in the rat esophagus (Aziz et al., 2002). As previously mentioned, blueberries differ in phytochemical content from strawberries and black raspberries in that their predominant tannins are PAs and not ETs. Two weeks prior to NMBA treatment, animals were placed on a control diet or diets containing 5 and 10% freeze-dried blueberries. At 25 weeks, animals on 5 and 10% blueberries produced no significant differences in tumor incidence, multiplicity, or size when compared to NMBA-treated controls. In addition, blueberries did not reduce the formation of NMBA-induced O6-methylguanine adducts in esophageal DNA when fed at 10% of the diet. The authors concluded that blueberries appear to lack components that inhibit the initiation and progression of NMBA-induced tumorigenesis in the rat esophagus (Aziz et al., 2002). It should be noted that although blueberries did not show anticancer properties in these studies, its consumption has been correlated with other health benefits such as anti-neurodegenerative properties and so on (Joseph et al., 1998, 2003).

## Human Studies

A survey of the literature revealed no published human clinical studies examining the anticancer effects of berries. However, data are available on the absorption, distribution, metabolism, and excretion of berry bioactives in humans obtained from foods, beverages, extracts, and as singly purified compounds (see the section “Bioavailability and Metabolism of Berry Bioactives,” later in this chapter).

A phase I study sought to examine tolerance for high dietary levels of freeze-dried berries that would be needed for chemoprevention studies (The James 2002/2003 Annual Report, Comprehensive Cancer Center, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, Ohio State University). Because the berries were well tolerated, studies are being designed to test the anticancer effects of these berries among individuals with precancerous lesions and at high risk for esophageal, colon, and oral cancers ([www.jamesline.com/patientsandpublic/research/index.cfm](http://www.jamesline.com/patientsandpublic/research/index.cfm)).

A human study (Moller et al., 2004) investigated the effects of black currant anthocyanins on the steady state level of oxidative DNA damage in mononuclear blood cells of 57 healthy human subjects, determined as strand breaks, as well as endonuclease III and formamidopyrimidine DNA glycosylase (Fpg). The baseline level of oxidative DNA damage was low and Fpg-sensitive sites increased during the intervention within the black currant anthocyanin group, whereas there were no differences between treatments in any of the DNA damage markers. The authors concluded that even large amounts of berry antioxidants did not decrease the already low steady state levels of oxidative DNA damage in healthy adequately nourished humans (Moller et al., 2004).

## DIETARY INTAKE OF BERRY BIOACTIVES

As previously discussed, phenolics are the predominant phytochemicals present in berry fruits. Unfortunately, because of the considerable number of factors that can modify their concentrations, reference food composition tables are not available. Their estimation in foods is also extremely challenging because of their wide structural diversity existing as different conjugated forms and complex polymeric nature, ill-defined structures, and unavailability of commercial standards. As a result, data on their dietary intake as well as data on their bioavailability and pharmacokinetics in humans are limited. Only partial data for certain phenolics, such as flavonols, have been published on the basis of direct food analysis or bibliographic compilations (Manach et al., 2004). In the United States, agencies such as the U.S. Department of Agriculture (USDA) have established databases in which the flavonoid contents of

selected foods, compiled from varying bibliographic sources, are available (USDA web site).

Studies have shown a high variability in polyphenol intake based on variations in individual food preferences. A diet consisting of several servings of fruit and vegetables per day can provide up to 1 g of phenolics consisting of the following: 16% flavonols, flavones, and flavanones; 17% anthocyanins; 20% catechins; and 45% PAs, ETs, and other "bioflavonoids" (Kuhnau, 1976). Among the berry bioactives, research has targeted individual data for specific classes of compounds. For example, consumption of flavonols has been estimated at 20–25 mg/day in the United States, Denmark, and Holland (Hertog et al., 1993; Justesen et al., 1997; Sampson et al., 2002). In Italy, consumption ranged from 5 to 125 mg/day, and the mean value was 35 mg/day (Pietta et al., 1996). In Finland, where high amounts of berries are eaten, anthocyanin consumption was found to be 82 mg/day on average, although some intakes exceeded 200 mg/day (Heinonen, 2001). Intake of phenolic acids ranged from 6 to 987 mg/day in Germany (Radtke et al., 1998).

Dietary burden, nature, and occurrence of specific classes of berry bioactives have been previously reviewed. These include anthocyanins (Clifford, 2000a), ETs (Clifford and Scalbert, 2000), PAs (Santos-Buelga and Scalbert, 2000), sterols (Piiroinen, 2000), hydroxybenzoic acid derivatives (Tomás-Barberán and Clifford, 2000), chlorogenic acid and other cinnamates (Clifford, 2000b), lignans and stilbenes (Cassidy et al., 2000), and flavonols, flavones, and flavanols (Hollman and Art, 2000).

### **BIOAVAILABILITY AND METABOLISM OF BERRY BIOACTIVES**

Several review articles have been published on the bioavailability and metabolism of phenolics (Scalbert and Williamson, 2000; Rechner et al., 2002; Manach et al., 2004, 2005a; Walle, 2004; Williamson and Manach, 2005). A wide body of studies have shown that although phenolics are the predominant phytochemicals in human diet, they are not necessarily the most active *in vivo*, either because they are poorly absorbed from the gut, highly metabolized, or rapidly eliminated. In addition, because of digestive and hepatic activities, the bioactivities of phenolic metabolites that are bioavailable in blood and target organs may differ significantly from their native forms. Hence, extensive knowledge of the bioavailability of phenolics is essential if their health effects are to be understood.

Because most phenolics are present in food in the form of esters, glycosides, or polymers that cannot be absorbed in their native form, they must be hydrolyzed by intestinal enzymes or by the colonic microflora before they can be absorbed, for example, into aglycons, which can then be

absorbed from the small intestine. When the gut microflora is involved, the efficiency of absorption is often reduced because the flora also degrades the aglycons that it releases and produces various simple phenolic and aromatic acids in the process. During the course of absorption, phenolics are conjugated (usually methylated, sulfated, and glucuronidated) in the small intestine and later in the liver, a metabolic detoxification process that facilitates biliary and urinary elimination. Because the conjugation mechanisms are highly efficient, aglycons are either absent in blood or present in very low concentrations after consumption of nutritional doses. Circulating phenolics are conjugated derivatives that are extensively bound to albumin (Scalbert and Williamson, 2000). Phenolics are able to penetrate tissues, particularly those in which they are metabolized, but reports on their ability to accumulate within specific target tissues are scarce (Manach et al., 2004). Phenolics are secreted via the biliary route into the duodenum, where they are subjected to the action of bacterial enzymes, especially  $\beta$ -glucuronidase, in the distal segments of the intestine, after which they may be reabsorbed. This enterohepatic recycling may lead to a longer presence of phenolics within the body.

The metabolism of the major classes of berry bioactives is discussed in the following subsections.

#### **Metabolism of Anthocyanins**

Among berry bioactives, the metabolism and bioavailability of anthocyanins in both human and animal models have been well studied (Prior, 2002). Human studies with anthocyanins have shown that albeit at low concentrations, they are detectable intact in human plasma (Cao and Prior, 1999; Cao et al., 2001; Milbury et al., 2002; Mazza et al., 2002; Bitsch, 2004). The elimination of plasma anthocyanins appears to follow first-order kinetics, and most anthocyanins were excreted in the urine within 4 hours of feeding (Milbury et al., 2002). Other studies have shown that anthocyanins and their metabolites are detectable in human urine after consumption of boysenberries (Cooney et al., 2004), strawberries (Felgines et al., 2003), and elderberries (Murkovic et al., 2001). The bioavailability of 15 structurally different anthocyanins from blueberry, boysenberry, black raspberry, and black currant in both humans and rats was investigated (McGhie et al., 2003). This study showed that intact and unmetabolized anthocyanins were detected in urine, although the relative concentrations of dosing varied, indicating that differences in bioavailability were due to variations in chemical structure. Anthocyanin metabolites and tissue distribution in digestive organs (stomach, jejunum, liver), kidney, and brain were studied in male Wistar rats fed with blackberry anthocyanins for 15 days (Talavera et al., 2005). Intact blackberry anthocyanins were detected in the stomach, while other organs (jejunum, liver, kidney) contained the anthocyanins in their intact and their

methylated and monoglucuronidated forms. Jejenum and blood plasma also contained anthocyanins in their aglycon forms. In the brain, the total anthocyanin content reached 0.25 nmol/g tissue (Talavera et al., 2005). Milbury et al. (2005) also demonstrated that berry anthocyanins cross the blood–brain barrier.

### Metabolism of Flavonols

Among berry flavonols, quercetin, the most ubiquitous flavonol in plant foods, is probably the most investigated. Hollman (1995, 1996) showed that quercetin is bioavailable in human plasma and demonstrated that glucosides of quercetin were more efficiently absorbed than quercetin itself, whereas the rhamnoglucoside (rutin) was less efficiently and less rapidly absorbed. The bioavailability of quercetin differs among food sources, depending on the type of glycosides they contain. For example, onions, which contain glucosides, are better sources of bioavailable quercetin than apples and tea, which contain rutin and other glycosides.

The presence of intact glycosides of quercetin in plasma had been debated, but it is now accepted that such compounds are absent from plasma after nutritional doses (Manach et al., 2005). On metabolism, phenolic acids can also be produced from flavonols by the gut microflora. Quercetin degradation produces mainly 3,4-dihydroxyphenylacetic, 3-methoxy-4-hydroxyphenylacetic (homovanillic acid), and 3-hydroxyphenylacetic acids (Manach et al., 2005).

### Metabolism of Tannins (Proanthocyanidins and Ellagitannins)

Assessment of the bioavailability and metabolism of tannins (PAs, ETs, and GTs) remains a challenge because of their ill-defined structures, lack of authentic standards, and lack of accurate data on their compositions in foods. Because of these challenges, there are few human studies reporting bioavailability of PAs and ETs (Seeram et al., 2004; Manach et al., 2005). Although the detection of PA dimers B1 and B2 in human plasma has been reported (Holt et al., 2002), studies done both *in vitro* and in animals have shown that polymerization greatly impairs intestinal absorption (Déprez et al., 2001; Donovan et al., 2002). An ET, punicalagin (MW 1084), was detected intact in rat plasma and was reported as the largest polyphenol observed *in vivo* (Cerdeira et al., 2003). Given the poor absorption of these molecules in their intact forms, it is possible that their biological effects may be attributable not only to direct actions of tannins themselves, but to the actions of some of their metabolites that can be more readily absorbed. PAs and ETs may be degraded into various phenolic and aromatic acids and other metabolites by the microflora (Cerdeira et al.,

2005a,b; Manach et al., 2005). ETs have been shown to release ellagic acid in human plasma (Seeram et al., 2004). Whereas the microbial metabolism of PAs has never been studied in humans after consumption of purified PA polymers (Manach et al., 2005), that of ETs has been reported (Cerdeira et al., 2004, 2005a,b). Therefore, further investigations into the degradation of PAs into microbial metabolites must be further evaluated in humans.

## CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, an overwhelming number of cell culture and animal studies suggest that berries may have immense potential for the prevention and treatment of cancer. Berry bioactives may act individually, additively, and synergistically to exert their chemopreventive properties. Because extrapolations cannot be made between *in vitro* and animal studies to humans, future clinical trials should be designed to investigate the potential of berries for the prevention and treatment of human cancers. In addition, further details on absorption, distribution, metabolism, and mechanisms of action of berry bioactives in humans are needed to determine effective dietary portions of berries. Whether bioactivities of berries are made stronger by the interactions of the many substances within a particular fruit, as well as in combination with phytochemicals from other fruits and vegetables, should be investigated. In addition, interactions of berries with prescription drugs and other herbal medicines, through their ability to modulate enzymes or cell receptors, should be investigated in carefully planned and controlled human clinical studies.

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