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Anthocyanins/Anthocyanidins and Colorectal Cancer: What Is Behind the Scenes?

Luís Fernando de Sousa Moraes[1,2], Xiaofei Sun[1], Maria do Carmo Gouveia Peluzio[2], Mei-Jun Zhu[*]

[1]School of Food Science, Washington State University, Pullman, WA 99164, USA

[2]Department of Nutrition and Health, Federal University of Viçosa, Viçosa – Minas Gerais, 36570-000, Brazil

[*]To whom correspondence should be addressed at School of Food Science, Washington State University, Pullman, WA 99164 USA; Phone: (509) 335--4016; Fax: (509) 335--4815; E-mail: meijun.zhu@wsu.edu

ABSTRACT

Colorectal cancer (CRC) is one of the most common cause of cancer death. Phytochemicals, especially anthocyanins/anthocyanidins (A/A), have gathered attention of the scientific community owing to its anti-inflammatory, antioxidant, and cancer-inhibitory properties. In this review, we discussed the possible mechanisms whereby A/A exhibit intestinal anticarcinogenic characteristics. Anthocyanins/anthocyanidins inhibit the pro-inflammatory NF-κB pathway, attenuate Wnt signaling and suppress abnormal epithelial cell proliferation. In addition, A/A induce mitochondrial-mediated apoptosis and downregulate Akt/mTOR (mammalian target of rapamycin) pathway. Furthermore, activation of AMP-activated protein kinase (AMPK) and SIRT1 also contributes to the anti-carcinogenic effects of A/A. Finally, downregulation of metalloproteinases (MMPs) by A/A inhibit tumor invasion and metastasis. In conclusion, A/A exert its anti-tumor effects against colorectal carcinogenesis via multiple mechanisms, providing

insights into the use of A/A as a natural chemopreventive intervention on major colorectal carcinogenic.

Keywords

polyphenols, anthocyanins, anthocyanidins, colorectal carcinogenesis, chemopreventive intervention, cancer prevention, signaling pathway.

Abbreviations

A/A   anthocyanins/anthocyanidins

ACF   aberrant crypt foci

Akt   protein kinase B

AMPK AMP-activated protein kinase

AOM   azoxymethane

APAF1   apoptotic protease-activating factor 1

Apc   adenomatous polyposis coli

ARE   anthocyanins-rich extract

ATM   ataxia telangiectasia mutated

ATR   ataxia telangiectasia and Rad3-related

Bax   Bcl-2-associated X protein

Bak   Bcl-2-killer

Bcl-2   B-cell lymphoma 2

Bcl-xL B-cell lymphoma-extra large

BH3   Bcl-2 homology 3

BW   body weight

CAD   caspase-activated deoxyribonuclease

caspases   cysteine aspartyl-specific proteases

COX-2 cyclooxygenase-2

CRB   crumb complex

CRC   colorectal cancer

DSS   dextran sodium sulfate

ERK   extracellular signal-regulated kinases

IFN-γ interferon gamma

IL   interleukin

iNOS   inducible nitric oxide synthase

Ki-67   marker of proliferation antigen Ki-67

MAPK mitogen activated protein kinase

MCT   medium-chain triacylglycerol

MMPs metalloproteinases

mTOR mammalian target of rapamycin

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NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells

p21 cyclin-dependent kinase inhibitor

p53 tumor suppressor protein

Par partitioning complex

PARP poly ADP-ribose polymerase

PCNA proliferating cell nuclear antigen

PGE_2 prostaglandin E2

pRB retinoblastoma protein

RNS reactive nitrogen species

ROS reactive oxygen species

Scrib scribble complex

SIRT1 sirtuin 1

SMAC second mitochondria-derived activator of caspases

STAT-3 signal transducer and activator of transcription 3

TNF-α tumor necrosis factor alpha

VEGF vascular endothelial growth factor

Wnt   Wingless and Int

XIAP   X-linked inhibitor of apoptosis protein.

1. Introduction

Colorectal cancer (CRC) appears to be the second most common cause of cancer death in the United States (Siegel et al., 2016). CRC affects more than one million patients every year worldwide (Ferlay et al., 2015). About 35% of overall cancer-related mortality is lifestyle-dependent (Doll and Peto, 1981). For instance, high dietary intake of fruits, vegetables, and whole grains have strongly sustained the inverse correlation between carcinogenesis and diet habits (Surh, 2003). Since inflammatory bowel disease (IBD) patients are predisposed to trigger the onset of colitis-associated CRC (Rhodes and Campbell, 2002) and only 15% of CRC occur due to inherited gene defect (Jackson-Thompson et al., 2006), it can be hypothesized, therefore, that inflammation management by antioxidants-rich food/extracts consumption could be a potential strategy to reduce the inflammation grade and, hence, prevent CRC onset. It is worth clarifying that although antioxidants-rich food/extracts intake itself is possibly not a recommended option to either treat or cure CRC, developing good dietary habits benefits towards intestinal health against the inflammation state.

In this sense, anthocyanins/anthocyanidins (A/A) have been emerged as promising compounds capable of promoting relevant health benefits in CRC (Shashirekha et al., 2015), owing to its known antioxidant and anti-inflammatory properties (Ravipati et al., 2012). Nevertheless, the important remaining question is how A/A exert its beneficial effects on CRC. Thus, in this review, we aimed at identifying the possible mechanisms whereby A/A exhibit intestinal

anticarcinogenic characteristics. In addition, the positive effects of other common polyphenols on colorectal carcinogenesis, although previously reviewed elsewhere (Juan et al., 2012; Kotecha et al., 2016; Priyadarsini and Nagini, 2012; Surh, 2003), are briefly introduced to contextualize and show the relevance of A/A as strong phytochemical compounds.

2. Polyphenols: Overview and Beneficial Effects on CRC

Dietary polyphenols or phenolic compounds are natural antioxidants present in plant-based foods, such as fruits, vegetables, tea, essential oils and their by-products (Zhang and Tsao, 2016), which can prevent the onset of chronic diseases, thus enhancing human health (Scalbert et al., 2007). Polyphenols can be mainly categorized into three different groups according to their chemical structures: phenolic acids, flavonoid and non-flavonoid compounds (Zhang and Tsao, 2016). **Table 1** summarizes the main findings related to the beneficial effects of bioactive compounds, mostly flavonoids, on colorectal carcinogenesis in mice.

The common signaling pathway underlying lower dysplasia and tumor incidence in polyphenols-treated mice is believed to be related to cell cycle arrest and decreased expression of inflammatory markers, such as tumor necrosis factor (TNF)-α, interferon gamma (IFN-γ), interleukin (IL)-6, and cyclooxygenase -2 (COX-2) (Table 1).

In particular, A/A are synthesized via the flavonoid pathway (Holton and Cornish, 1995) and has gained attention of the scientific community owing to its anti-inflammatory, antioxidant, and cancer-inhibitory properties (Bowen-Forbes et al., 2010). Among flavonoids, A/A provide strong electron-donating ability, which is comparable to carotenoids, one of the most remarkable natural

quencher of oxygen singlet (De Rosso et al., 2008). Additionally, A/A can be easily found and extracted from edible source plants (Cissé et al., 2012).

3. Anthocyanins/Anthocyanidins: Overview and Anticarcinogenic Effects

Anthocyanins comprise over 500 water-soluble compounds, naturally found at greater quantities in most colored fruits, vegetables, leaves and flowers (Wu et al., 2006; McGhie et al., 2003). Chemically, anthocyanins are classified as glycosides of polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium (Wu et al., 2007) and, thus, consist of two benzoyl rings (A and B) in between a heterocyclic ring (C), which in turn form the flavylium cation, as shown in **Figure 1**.

Anthocyanins most commonly present a tri-, di- or mono-saccharide unit. Hydrolyzed anthocyanins yield anthocyanidins and sugars (McGhie and Walton, 2007). Therefore, the so-called anthocyanidins or anthocyanins aglycones possess no sugar moiety attached to the molecular structure of the flavylium cation and are defined according to the substitute group – hydrogen atom, hydroxide or methoxy – that can be placed at the R1 and R2 positions (Figure 1). For instance, cyanidin is an anthocyanidin represented by the flavylium cation holding both –OH and –H substitutes at the R1 and R2 positions, respectively. Although several anthocyanidins have been properly identified, the anthocyanins mainly emerge from cyanidin, delphinidin, perlargonidin, peonidin, malvidin and petunidin (Jing et al., 2008).

To date, studies evaluating the effects of A/A on intestinal cancer in humans are sparse. In a previous study in CRC patients, 7-day treatment with a commercial anthocyanins-rich extract (ARE) from bilberry prior to tumor resection reduced the proliferation index, elucidated by lower

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Ki-67 expression, and increased the apoptotic index, observed by higher cleaved caspase-3 expression (Thomasset et al., 2009). Thus, further clinical trials should be more encouraged to provide results on A/A as a potential chemopreventive intervention.

On the other hand, the chemopreventive properties of A/A, indeed, have been successfully reported in rodent models for carcinogenesis (Hagiwara et al., 2001; Bobe et al., 2006). A 14-week supplementation with ARE from bilberry, chokeberry, and grape resulted in reduced number of colonic aberrant crypt foci (ACF, preneoplastic lesions of CRC) in AOM-induced CRC rats (Lala et al., 2006). Accordingly, Shi et al. (2015) revealed reduced tumor incidence and multiplicity (number of tumors per mouse) in AOM/DSS-promoted colorectal carcinogenesis in mice after 20-week supplementation with dietary lyophilized anthocyanins-rich strawberries. Cooke et al. (2006) have reported less intestinal adenomas in adenomatous polyposis coli $(Apc)^{Min}$ mice after 12-week treatment with either 0.3% of a commercial ARE from bilberry or the isolated anthocyanin type, cyanidin-3-glucoside. The number of intestinal tumors in Apc^{Min} mice was also decreased upon 7-week treatment with 0.5% of ARE from black soybean (Park et al., 2015). Likewise, Apc^{Min} mice consuming either a supplemented diet with anthocyanins-rich tart cherry, ARE from tart cherry in drinking water, or cyanidin for 10 weeks exhibited less and smaller cecal adenomas in comparison to mice under control diet or Sulindac (Kang et al., 2003), a non-steroidal anti-inflammatory drug, known to inhibit tumor progression (Boolbol et al., 1996). Positively, Apc^{Min} mice fed with different dosages of ARE from tart cherry in combination with Sulindac showed reduced total tumor area per mouse and tumor number when compared to Sulindac alone (Bobe et al., 2006).

The anticarcinogenic effect of A/A has also been evaluated *in vitro*. Cyanidin and ARE from tart cherry were able to induce a dose-dependent decrement in cell proliferation of both HCT-116 and HT-29 cells with no cytotoxic effects (Kang et al., 2003). Interestingly, cyanidin was even more potent in inhibiting cell growth in comparison with ARE from tart cherry. The IC50 for cyanidin, i.e. the concentration of cyanidin inducing a 50% reduction in cell proliferation, was much lower than that for anthocyanins (Kang et al., 2003). Anthocyanidins inhibited the proliferation in stomach, colon, lung, breast and central nervous system cancer cell lines, while anthocyanins at the same concentration could not inhibit above cell growth (Zhang et al., 2005).

Similarly, ARE from Chinese blueberry suppressed the proliferation of colon carcinoma cell lines, DLD-1 and COLO-205 cells (Zu et al., 2010). The IC50 and IC90 values of Chinese blueberry were much lower in relation to ARE from bilberry. It is worth commenting that, albeit both AREs consist mainly of the aglycone delphinidin, ARE from Chinese blueberry presents higher malvidin concentration and lower cyanidin percentage than ARE from bilberry (Zu et al., 2010). Thus, not only cyanidin but also other anthocyanidin types might also strongly contribute to the antiproliferative properties and pro-apoptotic activity of A/A.

Anthocyanins-rich extract from different sources may present distinct glycosylations, leading to different anticarcinogenic activities (Koide et al., 1997). Besides, the anthocyanin structure also influences its uptake and, therefore, affects its bioavailability (Kuntz et al., 2015). In this regard, Zhao et al. (2004) have investigated whether different anthocyanin profiles with expected distinct glycosylation would trigger similar or different responses on HT-29 cell proliferation. They found that the proliferation of HT-29 cells treated with ARE from grape, containing

acylated monoglycosides, was similarly inhibited (Zhao et al., 2004) when compared to treatment with ARE from bilberry, composed only by non-acylated anthocyanidins. Chokeberry-treated cells, receiving ARE containing only cyanidin derivatives, however, showed greater inhibition in relation to bilberry and grape (Zhao et al., 2004). Thus, different glycosylations attached to the anthocyanin structure will, indeed, influence the proliferation rate. Likewise, Jing et al. (2008) evaluated the cell growth inhibition of ARE from different sources and found that ARE from purple corn, consisting mainly of cyanidin-3-glucoside, induced the most potent growth inhibitory activity in HT-29 cell line, followed by chokeberry and bilberry. ARE from grape, however, was able to cause moderate growth inhibition.

The growth inhibitory effects of ARE are not only dependent on the source and glycosylation pattern of anthocyanins, but also on the storage time and maturity stage (Lewis et al., 1999; Blessington et al., 2010). At low storage temperatures (4 °C), starch is converted to sugars (Isherwood, 1976). In this situation, sugars function as signaling molecules and induce the upregulation of several genes involved in the anthocyanins biosynthesis pathway (Solfanelli et al., 2006), thus increasing anthocyanin contents. Anthocyanins contribute to the main portion of polyphenols in purple-fleshed potatoes (Charepalli et al., 2015). The anthocyanin concentration is increased in different purple potato extracts up to 60 days of storage at 3 °C (Madiwale et al., 2011). However, at 90 days of cold storage, most purple potato extracts presented lower anthocyanin contents. Such decrement might be one of the reasons why the antiproliferative effects of such extracts decreased after 3 months of storage (Madiwale et al., 2011).

Indeed, black raspberries from distinct harvest location, cultivar or maturity stage present different anthocyanin content, and, in consequence, the antiproliferative efficacy of black raspberry extracts on HT-29 cells is also influenced by their cultivars, production locations and maturation in a complex manner (Johnson et al., 2011).

4. Mechanisms Responsible for Anti-CRC Effects of Anthocyanins/Anthocyanidins

Anthocyanins/Anthocyanidins demonstrate strong preventive effects on intestinal tumor formation and development in preclinical animal models (Park et al., 2015; Shi et al., 2015). However, what might be the mechanisms by which A/A exert their protective properties on CRC? Accumulating studies have demonstrated the role of A/A in stimulating the expression of tumor suppressor genes and downregulating pro-oncogenic signals as well as controlling proliferation and apoptosis pathways (Forester et al., 2014; Charepalli et al., 2015).

4.1 Anthocyanins/Anthocyanidins Downregulate Pro-inflammation and Oxidation Pathways

Inflammation is basically a crucial protective response by the host defense against pathogens, harmful stimuli or damaged tissue. However, chronic excessive inflammation, as observed in IBD, has been markedly involved in different stages of tumor growth and colitis-associated CRC (Takeuchi and Akira, 2010). During carcinogenesis, the inflammatory microenvironment represses the host anti-tumor response, and thus, cancer-promoting immune activity stimulates tumor growth, angiogenesis, and metastasis (Grivennikov et al., 2009).

Over production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α in colitis-associated CRC can trigger signaling cascades that constitutively upregulate key pro-

inflammatory genes, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT3) (Szlosarek et al., 2006; Fan et al., 2013). The cross-talk between inflammatory signaling and Wnt/β-catenin pathway leads to β-catenin translocation towards the nucleus (Pramanik et al., 2015), which stimulates the downstream transcription of carcinogenic growth factors, cyclin D1 and c-Myc (Mishra et al., 2013; Clevers and Batlle, 2006), and therefore results in stem cell proliferation while blocking differentiation. Both intestinal epithelial cells and crypt stem cells fail to carry out appropriate cell division. Thus, besides inflammation and dysplasia, this process leads to ACF, and even carcinoma transition depending on the severity and duration of ulcerative colitis (Terzic et al., 2010).

Inflammatory cells release high amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are the well-known triggering substances of DNA damage and mutations (Meira et al., 2008), thereby worsening the disease prognosis and inhibiting earlier remission. Based on the chemical structure, A/A have strong ability for electron donation, which explains its unique antioxidant properties (Ali et al., 2016). The intracellular ROS activity was decreased in Caco-2 cells treated with cyanidin chloride or cyanidin-3-O-β glucopyranoside (Renis et al., 2008). Coherently, ARE from red wine inhibited the protein expression of inducible nitric oxide synthase (iNOS) in a dose-dependent manner in HT-29 cells, accompanied with reduced levels of both nitric oxide radical and protein tyrosine nitration, a biomarker of nitrosative stress (Nunes et al., 2013).

Apoptosis-dependent tumor surveillance mechanisms are altered in HT-116 cells under treatment with delphinidin due to inhibition of inflammatory NF-κB pathway (Yun et al., 2009). Besides inhibiting the phosphorylation and degradation of IκBα, delphinidin was able to suppress activation of I kappa B kinase α (IKKα), important to trigger IκBα activation in a dose-dependent way (Kumar Verma et al., 2012). As a result, phosphorylation of NF-κB/p65 was also inhibited by delphinidin, hence reducing the nuclear translocation of NF-κB/p65. This cascade of events subsequently led to lower transcriptional activation of inflammatory cytokines and, thus, induction of apoptosis and cell cycle arrest.

4.2 Anthocyanins/Anthocyanidins Induce Apoptosis

Anthocyanins/Anthocyanidins consumption has reduced intestinal tumor incidence and/or multiplicity in animal studies (Park et al., 2015; Silva et al., 2015). One of the mechanisms for such improvement is the fact that A/A act as antiproliferative agents *in vivo* through upregulation of malignant cell apoptosis mechanisms (Seeram et al., 2006).

4.2.1 Apoptosis introduction

Apoptosis is a highly complex event of programmed cell death characterized by morphologic changes, such as chromatin condensation and subsequent nuclear and DNA fragmentation (Kroemer et al., 2009). Two major apoptosis pathways are closely regulated to induce cell destruction: the extrinsic receptor-mediated pathway, represented by the activation of death domains and death effector domains on the cell surface; the intrinsic cytotoxic mitochondrial-mediated apoptosis, in which mitochondrial membrane permeabilization will lead to cysteine

aspartyl-specific proteases (caspases) activation (Parrish et al., 2013). Although both pathways will trigger effector caspases, most stimuli mainly induce apoptosis via mitochondrial outer membrane permeabilization (Lopez and Tait, 2015). Cells are stimulated to trigger cell death by apoptosis or necrosis when cells fail to repair DNA damage (Pommier, 2013). Once DNA lesions reach sufficient concentration, it activates cell cycle checkpoints and concomitant apoptosis machinery (Yoshida et al., 2008; Haince et al., 2007).

4.2.2 Cell death triggered by DNA damage

Given the potential devastating effects of gene instability, cells have developed a tight control of the main pathways of survival and death. Nevertheless, DNA damage in fact occurs during transcription and replication. The mechanisms involved in repairing the DNA are known as DNA damage response. In this context, topoisomerases I and II are key nuclear enzymes involved in the cell cycle progression and responsible to catalyze the phosphodiester backbone, thus allowing DNA unwinding for replication (Lord and Ashworth, 2012).

Anthocyanins-rich extract from bilberry and grape has also been described as topoisomerase inhibitors due to its ability to reduce topoisomerase I and II activity (Esselen et al., 2011; Habermeyer et al., 2005). However, it is noteworthy that A/A present no properties as topoisomerase poisons, since such compounds cannot stabilize the covalent DNA-topoisomerase intermediates of topoisomerase I or II, known as cleavable complex, which would also result in DNA lesions (Habermeyer et al., 2005). Interestingly, cyanidin and delphinidin, but not its isolated glycosides (cyanidin-3-glucoside and delphinidin-3-rutinoside, respectively) are effective in diminishing the catalytic activity of topoisomerases (Esselen et al., 2011;

Habermeyer et al., 2005). Compounds inhibiting topoisomerase function stimulate the formation of DNA single or double-strand breaks across the genome (Lord and Ashworth, 2012). Indeed, delphinidin acts as a topoisomerase inhibitor and, therefore, allows the increase in DNA strand breaks (Fritz et al., 2008). Thus, both DNA strand breaks and blocking lesions of DNA replication have been identified as downstream-apoptosis triggering lesions (Naumann et al., 2009).

The phosphatidylinositol 3-kinase-related kinases ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related) are crucial "sensors" of DNA lesions. Double-strand breaks and structural changes of the chromatin stimulate, respectively, ATM expression and its autophosphorylation, whereas stalled DNA replication forks mainly activate ATR (Caporali et al., 2004). Besides stimulating DNA strand breaks and inhibiting the catalytic activity of topoisomerases, A/A, such as cyanidin-3-O-β glucopyranoside and its aglycone, can upregulate the expression of ATM, which in turn stabilizes tumor suppressor p53 (Renis et al., 2008). The chemotherapeutic effects are observed by cell inhibitory proliferation, induction of DNA fragmentation and, hence, apoptosis.

4.2.3 Mitochondrial-mediated apoptosis

Programmed cell death can concomitantly be mediated by an intrinsic activation of a cascade involving both caspase and B-cell lymphoma 2 (Bcl-2) family of proteins (Brentnall et al., 2013). Increasing mitochondrial outer membrane permeabilization is the way by which Bcl-2 family protein determines the switch towards cell death rather than conferring survival functionality (Gavathiotis et al., 2008). Once activated over the apoptotic threshold by a diversity of cytotoxic

stress stimuli, such as DNA damage or growth factor deprivation, the initiator BH3 (Bcl-2 homology 3) inhibits Bcl-2, the anti-apoptotic cell guardian. In response, the pro-apoptotic effectors Bax (Bcl-2-associated X protein) and Bak (Bcl-2-killer) are then activated and undergo translocation from the cytosol to the mitochondrial outer membrane, where they are oligomerized and, hence, form pores (Czabotar et al., 2013). The release of apoptogenic factors, such as cytochrome C and second mitochondria-derived activator of caspases (SMAC), will trigger, respectively, the activation of apoptotic protease-activating factor 1 (APAF1) and the inhibition of X-linked inhibitor of apoptosis protein (XIAP). This process will activate caspase-9 and, consequently, the executioner caspase-3, -7 and -8 to carry out DNA fragmentation and degradation of cytoskeletal and nuclear proteins, thus favoring apoptosis (Li et al., 1997).

Although it remains unclear how exactly chromatin degradation takes place during apoptosis, it has been demonstrated that both caspase-activated deoxyribonuclease (CAD; also known as DNA fragmentation factor) and poly ADP-ribose polymerase (PARP)-regulated DNAS1L3, an endonuclease found in the endoplasmic reticulum, are key enzymes in this process (Errami et al., 2013). Once activated by the executioner caspases, specially caspase-3, CAD and DNAS1L3 contribute to internucleosomal DNA fragmentation. DNA fragmentation, known as "DNA ladder", is a key characteristic of apoptosis (Kello et al., 2016; Gorczyca et al., 1993). Anthocyanins-rich extract from different blueberry cultivars, containing mostly malvidin and peonidin glycosides, induces apoptosis in HT-29 cells as a result of increased caspase-3 activity and DNA fragmentation (Srivastava et al., 2007). In accordance, ARE from purple-shoot tea also mediates apoptosis in different colon cancer cell lines by activation of caspase-3 and its substrate PARP (Hsu et al., 2012).

Anthocyanins/Anthocyanidins might also have an important role in modulating such network of pro-apoptotic and anti-apoptotic proteins, since the expression of Bax mRNA is enhanced in HT-29 cells treated with ARE from bilberry (Wu et al., 2007). Although changes in Bcl-2 mRNA expression remain undetectable after treating cells with ARE from bilberry (Wu et al., 2007), delphinidin reduces the expression of Bcl-2 in HCT-116 cells in a dose-dependent manner with a concomitant augmentation in Bax expression, activation of caspase-9, -3 and -8, as well as the cleavage of PARP (Yun et al., 2009). In colon cancer stem cells, ARE from purple-fleshed potatoes and Java plum suppresses proliferation (Charepalli et al., 2015; Charepalli et al., 2016) by activating mitochondrial-mediated apoptotic pathway through elevating Bax and cytochrome C expression in a p53-independent way (Charepalli et al., 2015). In addition, activity of caspase-3 and -7, which will lead to DNA fragmentation, was also increased (Charepalli et al., 2016).

The mitogen activated protein kinase (MAPK) signaling pathways, mainly JNK/p38/ERK pathways, play a critical role in triggering apoptosis (Sui et al., 2014). Long term activation of ERK induces mitochondrial membrane disruption, leading to cytochrome C release and, thus, the activation of caspase-family proteins (Zhang et al., 2004; Cagnol et al., 2006; Tentner et al., 2012). Anthocyanins-rich extract from Meoru fruit inhibits cell growth and induces apoptotic cell death by activating phosphorylation of p38-MAPK and ERK with concomitant suppression of anti-apoptotic Akt and XIAP (Shin et al., 2009). Besides downregulating Akt, ARE from Meoru fruit inhibits the pro-tumorigenesis mTOR pathway through AMPKα1 activation, suggesting the anticancer effects (Lee et al., 2010).

4.3 Anthocyanins/Anthocyanidins Suppress Cancer Cell Proliferation by Inducing Cell Cycle Arrest

Anthocyanins/Anthocyanidins control malignant cell proliferation probably through cell cycle arrest as well (Lazze et al., 2004; Renis et al., 2008). Cell cycle is mainly highlighted by DNA replication (S phase) and chromosome segregation, resulting in the formation of two new daughter cells (M phase). Such key events are spaced by periods of cell preparation (G1 phase) and chromatin reorganization (G2 phase) (Salazar-Roa and Malumbres, 2017). Indeed, cells commonly follow a well-controlled cell cycle, regulated by the presence and activity of different cyclin-dependent kinases and their associated cyclins (Murray, 2004) or tumor suppressor proteins (Cordon-Cardo, 2004).

4.3.1 Cell cycle blockage by cyclin-dependent kinases

Besides DNA fragmentation and activation of pro-apoptotic pathways, ARE from different berries is able to induce overexpression of p21^{WAF1} and p27, two cyclin-dependent kinase inhibitors, known to restrain cell proliferation through induction of cell cycle blockage (Wu et al., 2007; Hsu et al., 2012). Anthocyanins-rich extract from chokeberry showed antiproliferative effects in HT-29 cells through dual cell cycle arrest at G0/G1 and G2/M phases, due to overexpression of p21^{WAF1} and p27^{KIP1} and downregulation of cyclins A and B (Malik et al., 2003). Such outcomes might be attributed to a specific anthocyanin, since almost 70% of the total anthocyanins present in the chokeberry extract are cyanidin-3-galactoside. Consistently, pure delphinidin blocked cell cycle at G2/M phase in HT-116 cells (Yun et al., 2009). The cellular mechanism responsible to inhibit COLO 320DM cell proliferation by ARE from purple-

shoot tea was mainly through cell cycle blockage (Hsu et al., 2012). Notably, cells are blocked at the G1 phase and accompanied concomitant decrease in S phase. Besides, cyclins D1 and E expression was downregulated in a dose-dependent manner (Hsu et al., 2012). In colon cancer stem cells, which have been reported to possess an important role in forming and sustaining tumor expansion (Barker et al., 2009), ARE from purple-fleshed potato reduce cell proliferation by downregulating β-catenin levels, which in turn decrease the levels of its downstream proteins, cyclin D1 and c-Myc (Charepalli et al., 2015), both involved in cell cycle blockage (Santoni-Rugiu et al., 2000).

As previously discussed, different A/A behave distinctively on cell proliferative control. Hypothetically, their effects on cell cycle progression or arrest pathways might also differ. Indeed, Caco-2 cell growth was more suppressed by cyanidin chloride when compared with cyanidin-3-O-β glucopyranoside (Renis et al., 2008). Furthermore, both anthocyanins were able to induce DNA fragmentation, but only cyanidin chloride treatment induced a decrease in ROS production. The ATM/p53 pathway, known to disturb cell cycle and prevent cell proliferation through the activation of p21, was only upregulated by cyanidin chloride treatment, which suggest that these anthocyanins might have different effects on cell cycle blockage.

4.3.2 Tumor suppressor proteins as cell cycle arrest inductors

It is important to highlight that tumor suppressor proteins, such as p53 and retinoblastoma protein (pRB), have critical roles in blocking abnormal cell proliferation; their mutations may lead to uncontrolled cell division (Cordon-Cardo, 2004). Most sporadic CRC development is owing to mutations in the *Apc* tumor suppressor gene (Fearon, 2011), which mediates β-catenin

degradation (Kaler et al., 2009), thus contributing to adenoma-carcinoma sequence (Tarmin et al., 1995).

Anthocyanins-rich extract from Illawarra plum was effective in reducing HT-29 cell proliferation associated with cell cycle blockage at the S phase and induction of p53-independent apoptosis and necrosis (Symonds et al., 2013). Additionally, ARE treatment resulted in telomere shortening and decreased expression of telomerase reverse transcriptase, indicating ARE functions as a telomerase inhibitor. Telomerase inhibition followed by reduction in telomere length is an early event in the apoptosis pathway that will lead to restrained cell proliferation, disrupted cell cycle and subsequent apoptosis cell death (Boklan et al., 2002). Moreover, most HT-29 cells treated with ARE from Illawarra plum exhibited high numbers of cytoplasmic vacuoles, suggesting cell autophagy (Symonds et al., 2013). Interestingly, the expression of sirtuin 1 (SIRT1), which has been demonstrated to trigger autophagy (Lee et al., 2008) and inhibit β-catenin pathway (Firestein et al., 2008), was also increased with Illawarra plum extract treatment (Symonds et al., 2013).

4.4 Anthocyanins/Anthocyanidins Inhibit CRC Metastasis Through Suppressing Matrix Metalloproteinases

The extracellular matrix is composed of proteins and proteoglycans, which are responsible to keep cell attachment, thus providing structural integrity to tissues (Cox and Erler, 2011). The human matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases ascribed to be involved in inflammatory tissue destruction and capable of degrading basement membrane collagen (Vandenbroucke and Libert, 2014). Accumulating evidence suggests its role

in the pathogenesis of IBD (Matusiewicz et al.,2014; Nighot et al., 2015) and, hence, in cancer development (Egeblad and Werb, 2002). A tumor cell can metastasize to other organs if the components of the extracellular matrix are degraded by MMPs. Therefore, MMP suppression might be one of the promising targets for cancer therapy (Gialeli et al., 2011). In this context, A/A exhibit anti-invasive activities by suppressing the expression of MMP-2 and MMP-9 in a dose-dependent manner (Shin et al., 2011; Yun et al., 2010).

It is important to highlight that albeit MMPs are notably related to invasion and metastasis, late events in cancer progression, studies have also emphasized its functions in immunity, such as the intertwine between MMPs and inflammation. Matrix metalloproteinases can directly or indirectly mediate the expression of several inflammation-related cytokines or pathways (Nelissen et al., 2003). For instance, the pro-inflammatory IL-1β precursor needs to be cleaved to become active (Yazdi and Ghoreschi, 2016). MMP-2, -3 and -9 can break down and activate the IL-1β precursor (Schonbeck et al., 1998). Furthermore, MMPs (MMP-3, -7, -9, -12, -17) can turn latent TNF-α into bioavailable TNF-α (Haro et al., 2000; Churg et al., 2003), which results in the pro-tumorigenesis NF-κB pathway activation (Ferrari et al., 2016). Anthocyanins/Anthocyanidins can indeed contribute to a dual beneficial effect on tumor cell growth: reducing the expression of various pro-metastasis MMPs and additionally suppressing pro-inflammatory mechanisms via MMPs downregulation (Chen et al., 2006).

There is a positive regulation between MMPs and the Akt/mTOR signaling pathway, which has been reported elsewhere in CRC cells (Li, et al., 2016; Zhang et al., 2015). In addition, an invasive growth of CRC cells is observed when enhanced expression of MMPs takes place due

to deactivation of AMPK (Banskota et al., 2015) and posterior phosphorylation and activation of Akt/mTOR (Zhan et al., 2017). Although the role of mTOR and its downstream effectors on metastasis invasiveness activation remains speculative (Zhan et al., 2017), it can be hypothesized that inhibition of MMPs, hence activation of AMPK and inhibition of Akt/mTOR could reduce the invasive phenotype in CRC cells. Anthocyanins-rich extract from Meoru fruit suppresses Akt/mTOR phosphorylation, in addition to triggering apoptosis, by stimulating AMPKα1 activation (Lee et al., 2010), further suggesting the potential role on metastasis prevention.

The suppressing properties of A/A on cell growth and invasiveness have also been associated with modulation of tight junction proteins, including claudin-1, -3 and -4 (Shin et al., 2011). Such claudins are crucial transmembrane proteins found to be overexpressed in CRC (Mees et al., 2009). Interestingly, besides suppressing claudin-1, -3 and -4 in HCT-116 cells, ARE from Meoru fruit improved the tightness of tight junctions (cell-cell adhesion), as measured by increased transepithelial electrical resistance in a concentration-dependent manner (Shin et al., 2011), thus reducing cell invasion. Moreover, restoration of functional tight junction proteins has recently been reported to be related to apico-basal polarity proteins, which might influence where the tight junctions will be formed (Borovski et al., 2016). Thus, future studies should also address the role of A/A on cell invasion by modulating such tight junction-associated protein complexes, i.e. the crumb (CRB) complex, the partitioning defective (Par) complex, and the scribble (Scrib) complex.

5. Conclusions

As the third lethal cancer in the United States, the need of new preventive approaches for CRC has become increasingly crucial. In this sense, bioactive compounds would be an easy dietary strategy to provide a therapeutic and nutritional alternative for CRC. Specially, growing evidence shows that A/A have beneficial effects on the management of CRC development. Thus, this review summarizes current literatures on anti-CRC health-promoting effects of A/A and their underlying mechanisms (**Figure 2**). Mainly, A/A mediate colorectal carcinogenesis via stimulation of apoptosis pathways, cell cycle arrest and inhibition of metastasis, suppression of cell proliferation, as a result of downregulation of inflammatory and oxidative mechanisms. Most *in vitro* and *in vivo* studies, in fact, indicate the chemopreventive properties of A/A. However, due to the lack of human studies assessing the beneficial effects of anthocyanins-rich food/extracts on CRC, the results are still unclear at clinical level. In addition, more studies are needed on the interaction between A/A and the host gut microbiota, in order to assess how the gut microbiota-derived anthocyanin metabolites influence the bioavailability of A/A, carcinogenesis, and growth of cancer cells, as well as the onset and development of CRC in animal models and human studies.

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TABLE 1 Main findings related to the beneficial effects of bioactive compounds on azoxymethane

and DSS-induced colorectal carcinogenesis in mice

Bioactive Compound (Group)	Carcinogen	Mice and Groups	Time of Treatment	Main Findings	Comments and Limitations	Reference
Auraptene and collinin (Non-flavonoid coumarin)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 1% for 7 days on day 8.	Male ICR mice (n = 10, 6 weeks old). Cancer-induced: 1) Basal diet; 2) Basal diet + 0.01% auraptene; 3) Basal diet + 0.05% auraptene; 4) Basal diet + 0.01% collinin; 5) Basal diet + 0.05% collinin.	20 weeks. Auraptene and collinin treatment started one week after DSS administration.	↓ Incidence of adenomas and adenocarcinomas in all groups x group 1; ↓ Total tumor multiplicity in groups 3 and 5 × 1; ↓ Inflammation score in groups 3 and 5 × 1; ↓ PCNA-labeling index in all groups x group 1; ↑ Apoptotic index in groups 2, 4 and 5 × 1; ↓ Expression of COX-2 and iNOS in all groups x group 1, except group 4 for COX-2; ↓ Expression of nitrotyrosine in groups 3 and 5 × 1.	1) Usually, results with the highest doses for both compounds were better when compared to the lowest dose. 2) No adverse effects were noticed for both dosages and compounds.	(Kohno et al., 2006)
Epigallocatechin gallate and Polyphenon E (Flavonoid)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 2% for 7 days on day 8.	Male ICR mice (n = 10, 5 weeks old). Cancer-induced: 1) Control; 2) 0.01% epigallocatechin gallate; 3) 0.1% epigallocatechin gallate; 4) 0.01% polyphenon E; 5) 0.1% polyphenon E.	17 weeks. Epigallocatechin gallate and polyphenon E treatment started 1 week after DSS administration.	↔ Colon length and tumor incidence between the groups; ↓ Multiplicity of colonic adenocarcinomas in groups 2, 4 and 5 × 1; ↓ Inflammation score in all groups x 1; ↓ Expression levels of COX-2 in all groups x 1; ↓ Expression of TNF-α, IFN-γ, IL-6 and IL-12 in all groups x 1.	1) The reduction on inflammatory cytokines was significantly better in group 3 when compared to the others.	(Shirakami et al., 2008)
Resveratrol (Non-flavonoid stilbene)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 1% on day 8 for 7 days + 14 days of normal water. Cycle was repeated twice.	Male and female C57BL/6 mice (n = 10, 8--12 weeks old). Cancer-induced: 1) AIN-93M diet; 2) AIN-93M + 300 ppm resveratrol.	10 weeks. Resveratrol treatment started on day 8.	↓ Tumor incidence in group 2 × 1; ↓ Tumor multiplicity in group 2 × 1.	1) Group 2 exhibited lower tumor size when compared to 1, although not significantly.	(Cui et al., 2010)
Isorhamnetin, myricetin, quercetin and rutin (Flavonoid)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 2% for 7 days on day	Male FVB/N mice (n = 12, 6 weeks old). Cancer-induced: 1) AIN-93G diet; 2) AIN-93G + 552 ppm isorhamnetin; 3) AIN-93G +556 ppm myricetin; 4) AIN-93G + 591 ppm quercetin; 5) AIN-93G +	14 weeks. Flavonols treatments started 3 days after DSS administration was ended (day 17).	↓ Morbidity in groups 2 and 4 × 1; ↓ Tumor multiplicity, tumor burden and tumor size in group 2 × 1;	1) Flavonols were added at equimolar concentrations; 2) FVB/N mice are sensitive to AOM/DSS, thus requiring	(Saud et al., 2013)

	8.	1099 ppm rutin.		↓ Inflammation grade in group 2 × 1; ↓ Number of Ki-67-positive cells in group 2 × 1; ↓ β-catenin accumulation in the nucleus in group 2 × 1.	only one cycle of DSS; 3) No beneficial effects in groups 3, 4 and 5.	
Oroxylin A (Flavonoid)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 2.5% on day 8 for 7 days + 14 days of normal water.	Male and female C57BL/6 mice (6–8 weeks old). Cancer-induced: 1) Saline; 2) 50 mg oroxylin/kg BW; 3) 100 mg oroxylin/kg BW; 4) 200 mg oroxylin/kg BW.	14 weeks. Oroxylin A treatment started one week prior to AOM injection.	↔ Colon length between the groups; ↓ Inflammatory score in groups 3 and 4 × 1; ↓ Tumor number and tumor burden in all groups x 1; ↓ Tumor size in groups 3 and 4 × 1; ↓ Number of Ki-67-positive cells in all groups x 1; ↓ Expression of IL-6 and IL-1β in all groups x 1.	1) Oroxylin A exhibited antiproliferative and pro-apoptotic effects.	(Yang et al., 2013)
Carnosic acid (Non-flavonoid diterpene)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 2% on day 8 for 7 days + 14 days of normal water.	Male A/J mice (4 weeks old). Cancer-induced: 1) Normal diet (n = 7); 2) high-fat diet (n = 7); 3) high-fat diet + 0.01% carnosic acid (n = 8); 4) high-fat diet + 0.02% carnosic acid (n = 5).	11 weeks. Carnosic acid treatment started on day 1.	↓ BW and epididymal fat weight in group 4 × 2; ↓ Total number of tumors in groups 4 and 3 × 2; ↔ Tumor multiplicity and size between the groups; ↔ p-Akt and STAT-3 between the groups; ↓ Cyclin-D1 and Bcl-xL in group 4 × 2.	1) High-fat diet accelerates tumor development (higher total number of tumors in group 2 × 1).	(Kim et al., 2014)
Isoliquiritigenin (Flavonoid)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 2% on day 8 for 7 days of normal water. Cycle was repea	Male BALB/c mice (6 weeks Cancer-induced (n = 20): 1) Saline isoliquiritigenin/kg BW; 3) isoliquiritigenin/kg BW; 4) isoliquiritigenin /kg BW.	13 weeks. Isoliquiritigenin started one week prior injection.	↓ Colon weight-to-length ratio and histological score in groups 3 and 4 × 1; ↓ Tumor multiplicity and size in groups 3 and 4 × 1; ↓ IL-6, PGE₂ levels in groups 3 and	1) Group 4 exhibited the lowest tumor incidence; 2) IL-6 promotes inflammation activation of STAT3 pathway.	(Zhao et al., 2014)
Tangeretin (Flavonoid)	1) AOM – 20 mg/kg BW on day 1; 2) DSS 2% for 7 days on day 8.	Male ICR mice (n = 9, 6 w Cancer-induced: 1) MCT suspe Blank emulsion; 3) 100 mg tan MCT suspension/kg BW; 4) tangeretin in blank emulsion/kg B	12 weeks. Tangeretin treatm one week prior to AOM inje	↔ Body weight between the groups; ↔ Colon length and tumor incidence; ↓ Colon weight-to-length ratio and multiplicity of adenomas in group 4 × 2; ↓ Expression of COX-2 and VEGF in group 4 × 2;	1) No signs of toxicity; 2) Group 4 exhibited the lowest tumor incidence; 3) Goblet cells were better preserved in group 4; 4) Efficacy of tangeretin to attenuate CAC growth was	(Ting et al., 2015)

					better with the emulsion-based delivery system.	
				↓ Expression of PCNA and β-c group 4 × 2.		
Curcumin (Flavonoid)	1) AOM – 25 mg/kg BW on day 1; 2) DSS 2% on day 8 for 5 days + 16 days of normal water. Cycle was repeated once with DSS 1%.	Female BALB/c mice (n = 10, 5 weeks old). Cancer-induced: 1) normal protein diet; 2) high protein diet; 3) high protein diet + 0.02% curcumin.	8 weeks. Curcumin treatment started on day 1.	↓ Body weight and food intake in groups 3 and 2 × 1; ↑ Number of tumors in group 2 × 1; ↓ Number of tumors in group 3 × 2; ↓ Expression of COX-2 and iNOS in group 3 × 2; ↔ Plasma levels of TNF-α and nitric oxide; ↓ Fecal NH₄⁺/NH₃ in group 3 × 2.	1) DSS dosage was reduced due to severe disease activity; 2) Curcumin attenuates the effects of a high protein diet on CAC development.	(Byun et al., 2015)

↔ No difference; ↑ higher; ↓ lower. Akt, protein kinase B; AOM, azoxymethane; Bcl-xL, B-cell lymphoma-extra large; BW, body weight; COX-2, cyclooxygenase-2; DSS, dextran sodium sulfate; IFN-γ, interferon gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; Ki-67, marker of proliferation antigen Ki-67; MCT, medium-chain triacylglycerol; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PCNA, proliferating cell nuclear antigen; PGE₂, Prostaglandin E2; STAT-3, signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.
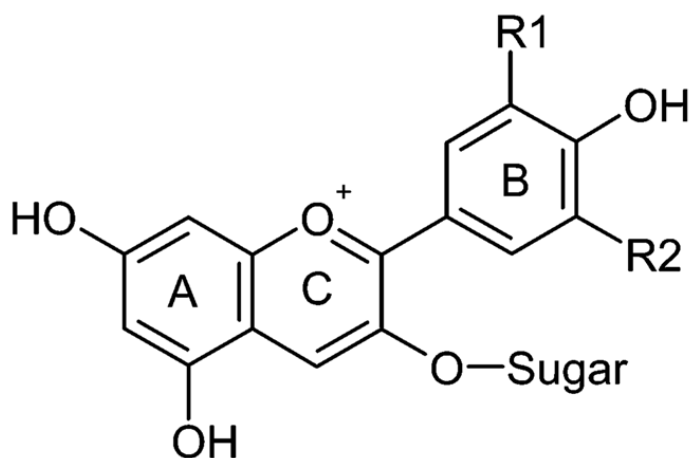
Figure 1. Chemical structure of the flavylium cation (left). The main anthocyanidins are formed according to the specific substitutes at R1 and R2 positions (right). Anthocyanins, in turn, mostly present tri-, di or mono-saccharide unit incorporated into the anthocyanidin structure.
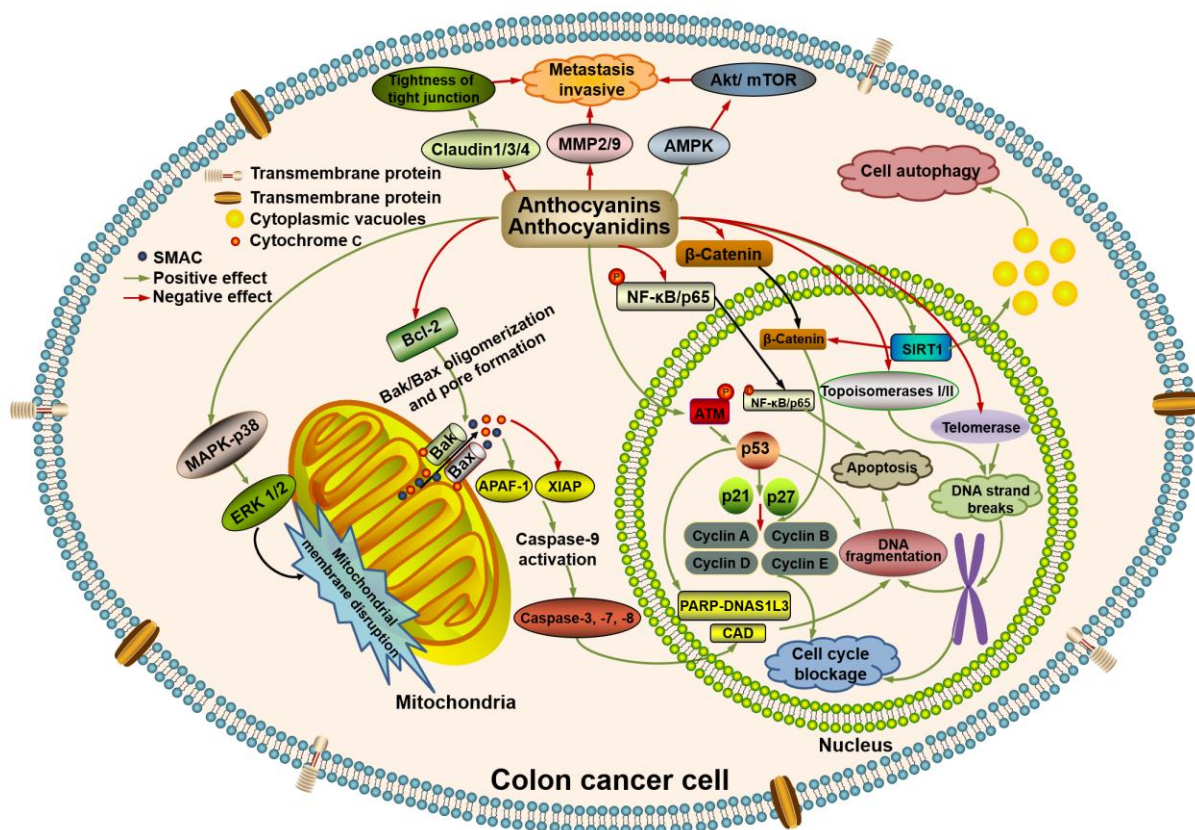
Figure 2. The possible anticarcinogenic mechanisms of anthocyanins/anthocyanidins in colon cancer cells. Anthocyanins/anthocyanidins (A/A) inhibit the pro-inflammatory NF-κB signaling pathway and β-catenin translocation to stimulate cell cycle blockage. A/A act as topoisomerase inhibitors and stimulate DNA strand break responses. Furthermore, A/A phosphorylate ATM to trigger DNA fragmentation and cell cycle blockage. A/A disrupt mitochondrial membrane to induce apoptosis. Concomitantly, A/A enhance tight junction formation, suppress metastasis invasiveness, and increase cell autophagy. Akt, protein kinase B; AMPK, AMP-activated protein kinase; APAF1, apoptotic protease-activating factor 1; ATM, ataxia telangiectasia mutated; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; BH3, Bcl-2 homology 3; CAD,

caspase-activated deoxyribonuclease; ERK, extracellular signal-regulated kinases; MAPK, mitogen activated protein kinase; MMPs, metalloproteinases; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; SIRT1, sirtuin 1; SMAC, second mitochondria-derived activator of caspases; XIAP, X-linked inhibitor of apoptosis protein. The green arrows indicate demonstrated effects. The black dashed arrows indicate the potential effects. The red lines indicate negative effect.

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