Orientin: A C-Glycosyl Flavonoid that Mitigates Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is one of the most frequent malignancies, causing human deaths in large numbers around the world. It can be prevented by using chemotherapeutic drugs; however, the available drugs have shown systemic toxicity and drug resistance, which makes the treatment a challenging issue. The use of flavonoid-rich sources may effectively reduce the risk of colorectal cancer. Orientin, a C-glycosyl flavonoid exists in diverse medicinal flora, such as *Aspalathus linearis*, *Ocimum tenuiflorum*, *Passiflora*, and *Phyllostachys* species, and it has been shown to have beneficiary effects in treating cancers, cardiovascular diseases, and neurodegenerative disorders. Recent preclinical validation studies on orientin have evidently shown its anti-carcinogenic effect against human colorectal adenocarcinoma cells and carcinogen-induced CRC albino Wistar rat models. Orientin reinstates the antioxidants to put forth their scavenging mechanism and limits the activation of phase 1 enzymes. Orientin induces mitochondrial intrinsic apoptosis in CRC cells, and thereby actively interrupt cell proliferation and inflammatory signaling pathways without disturbing the normal tissue. Research reports have reported the attenuating effects of orientin on aberrant crypt foci progression in cancer-bearing animal, and it results in a significant suppression of pre-neoplasia to malignant neoplasia transformation. Orientin also suppresses NF-κB and the associated inflammatory cytokines, and thereby ameliorates inducible nitric oxide synthase and cyclooxygenase-2 expression in 1,2-dimethylhydrazine rat models. Thus, this chapter emphasizes the therapeutic effects of flavonoids with a special focus on orientin against CRC. Also, it summarizes our understandings about the molecular mechanisms behind orientin-mediated cancer prevention.

Keywords

Colorectal cancer · Orientin · Chemoprevention · Apoptosis · Cell proliferation

1.1 Introduction

Chemoprevention involves the inhibition or delay in the development of neoplasia by interrupting the instigation of neoplasia as well as transformation before malignancy. Colorectal cancer is probably a preventable cancer. The chemoprevention of colorectal cancer (CRC) has been the spotlight of research for three decades, which intends to thwart or delay the commencement of cancer through the deterioration or anticipation of colonic adenomas (Ricciardiello et al. 2016). Chemotherapy is highly recommended for colorectal cancer patients as palliative and adjuvant chemotherapy. It also depends on the tumor staging of CRC. Adjuvant chemotherapy is optional for patients under the stage III and in some cases for stage II patients with a chance of recurrence after the surgery. Palliative chemotherapy is optional for the stage IV patients, when the cancerous cells spread from the colon to other organs and tissues that are even far apart. 5-Fluorouracil, irinotecan, capecitabine, oxaliplatin, tipiracil, and trifluridine are some of the common chemotherapeutic agents that are used against CRC (Idrees and Tejani 2019; Akhtar and Swamy 2018).

A huge number of chemopreventive agents demonstrated promising results in preclinical models; however clinical trials have succumbed to contradictory outcomes. The detrimental effects of some of the clinically proven antitumor compounds boost up death cases and accentuate a vital need for novel and nontoxic chemopreventive agents (Youns and Hegazy 2017; Bhatia and Nair 2018; Roy et al. 2018). Many known natural compounds are notorious to exert significant inhibitory action against aberrant signaling pathways involved in carcinogenesis. Furthermore, the natural compounds can be obtainable readily. They are cost-effective and harmless with both protective and health-giving potential against cancers (Arivalagan et al. 2015; Bhatia and Nair 2018). The compounds of natural origin, such as dietary agents, have been of immense interest due to the development of novel and innovative therapeutic agents for cancer (Karthi et al. 2016; Tuorkey 2016; Roy et al. 2018). In this regard, medicinal plants have an immense potential of providing natural agents, i.e., secondary metabolites that are effective in treating several types of cancers. Among plant-derived metabolites, flavonoids are found to have several medicinal importance (Chang et al. 2018; Anwar et al. 2019; Jain et al. 2019). Flavonoids like umbelliferone, luteolin, orientin, and eriodictyol have been shown to have considerable antiproliferative activities against cancerous cells. Reports have shown the attenuating effects of orientin on aberrant crypt foci (ACF) progression in cancer-bearing animal, and it results in a significant suppression of preneoplasia to malignant neoplasia transformation (Muthu et al. 2016; Mariyappan et al. 2017; Thangaraj et al. 2018). Orientin has also been shown to suppress NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and the associated inflammatory cytokines and thereby ameliorates inducible nitric oxide synthase and cyclooxygenase-2 expression in 1,2-dimethylhydrazine rat models (Hamiza et al. 2012a, b; Thangaraj et al. 2018). Thus, this chapter emphasizes the therapeutic effects of flavonoids with a special focus on orientin against CRC. Also, it summarizes our understandings about the molecular mechanisms behind orientin-mediated cancer prevention.

1.2 Flavonoid's Role in Cancer Prevention

Flavonoids possess the potential of regulating multiple carcinogenic processes, such as apoptosis, angiogenesis, tumor differentiation, and cell proliferation. The flavonoid-induced alterations in kinase activity have a strong relationship with apoptosis, tumor cell proliferation, and invasion. Some of the dietary flavonoids have displayed in vivo antitumor activity and repress inflammation, proliferation, vascularization, and metastasis (Kumar and Pandey 2013; Panche et al. 2016; Tungmunnithum et al. 2018). Epidemiological studies on colon cancer proposed that some flavonoids that prevent colon cancer may enhance therapeutic efficiency by regulating colon tumor cell proliferation and survival signaling pathways (Zamora-Ros et al. 2015; Thangaraj et al. 2018). Flavonoids block several steps in carcinogenesis, namely tumor cell transformation, invasion, and metastasis (Chahar et al. 2011). A positive correlation exists between a flavonoid-rich diet and lowers the risks of colon cancer. Several flavonoids, such as umbelliferone, luteolin, and eriodictyol have been shown to exhibit superior inhibitory activities against colorectal cancer cells (Manju and Nalini 2010; Muthu et al. 2016; Mariyappan et al. 2017). Naturally available flavonoids most commonly exist as O- or C-glycosides. C-Glycosylflavones are the major class of flavonoids, which imparts the dark-yellow color to flowers of Leguminosae family members. C-Glycosides are well known for their diverse pharmacological potentials, including antioxidant, antiinflammatory, antimicrobial, and antitumor activities.

1.3 About Orientin

1.3.1 Chemistry

The IUPAC name of orientin is 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-8-[(2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one. Orientin is a water-soluble C-glycosyl flavonoid with the molecular formula $C_{21}H_{20}O_{11}$, and its molecular weight is 448.38 g/mol. In general, C-glycosyl flavonoids possess C_6–C_3–C_6 flavone skeleton, in which two aromatic rings are attached by three carbons cyclized with oxygen, having one to several glucoside units. Orientin has a 15-carbon skeleton, consisting of two phenyl rings (A and B); in specific O-phenolic hydroxyl group at 3′ of B ring and a heterocyclic ring exemption of a C-glycosides linked to glucose of the A ring (An et al. 2015). It comprises phenol groups alongside one ketone group and two ether groups. It is the 8-C glucoside of luteolin, substituted by a β-D-glucopyranosyl moiety at position 8.

1.3.2 Natural Occurrence

Several medicinal plants are the source of orientin. Some of the most widely explored medicinal plants for orientin include *Ocimum sanctum* (holy basil) (Devi

et al. 2004), *Aspalathus linearis* (rooibos tea) (Koeppen et al. 1962), *Phyllostachys nigra* (bamboo leaves), *Passiflora edulis* (passion flowers, juice and peel of passion fruit), *Trollius chinensis* Bunge (Golden Queen), *Linum usitatissimum* (flax) (Dubois and Mabry 1971), *Jatropha gossypifolia* (bellyache bush), *Commelina communis* (dayflower) (Shibano et al. 2008), *Euterpe oleracea* Mart. (acai palm) (Gallori et al. 2004), *Ascarina lucida* (Soltis and Bohm 1982), *Roscoea capitata*, *Celtis africana* (white stinkwood) (Perveen et al. 2011), *Croton zambesicus* (lavender croton) leaves (Wagner et al. 1970), *Cajanus cajan* (pigeon pea) leaves (Pal et al. 2011), *Thlaspi arvense* (field penny-cress) (Pang et al. 2013), *Fagopyrum esculentum* (buckwheat) (Krahl et al. 2008), *Trigonella foenum-graecum* L. (fenugreek) oils (He et al. 2014), *Clinacanthus nutans* (Sabah snake grass) leaves (Chelyn et al. 2014), and *Polygonum orientale* L. (Li et al. 2017a).

1.3.3 Pharmacological Properties

Orientin has been testified to possess numerous medicinal properties, such as anti-aging, antiviral, antibacterial, antiinflammation, cardioprotective, antinociceptive, radioprotective, and neuroprotective effects. Orientin inhibits esophageal cancer (EC-109) cell development in a time-dependent and dose-dependent way. It has been found to downregulate the anti-apoptotic B-cell lymphoma 2 (Bcl-2) expression and induce early apoptosis in EC-109 cells. Orientin was found to have higher antitumorigenic effect, when compared to vitexin (an apigenin flavone glycoside). The researchers have doubted on the associated OH-groups at the 3′- and 4′-position of the B ring in orientin for the increased effect. Due to the presence of a single OH-group at the 4′-position of the B ring in vitexin, its was believed to be less effective (An et al. 2015). Orientin inhibited the multiplication of HeLa (cervical cancer) cells in a concentration-dependent way and stimulated apoptosis. It reduced anti-apoptotic Bcl-2 and enhanced pro-apoptotic Bax (bcl-2-like protein 4) protein levels in HeLa cells. Also, it instigated the proteolytic stimulation of protease enzymes, i.e., caspases (Guo et al. 2014). Orientin induces apoptosis and inhibited the proliferation of breast cancer (MCF-7) cells in a time- and dose-dependent way (Czemplik et al. 2016). However, so far, none of the information validates the influence of orientin against CRC. Further, the precise molecular mechanisms of action of cell inhibitory activities induced by orientin still remain unclear.

1.4 Experimental Colorectal Carcinogenesis

1.4.1 HT29 (Human Colorectal Adenocarcinoma) Cell Line

HT29 is a human colon adenocarcinoma with epithelial morphology. This cell line was firstly obtained from a Caucasian woman (aged 44 years) suffering from a cancer of the colon (Fogh and Trempe 1975). HT29 cells contain unique features, such as microvilli, microfilaments, lipid droplets, smooth and rough endoplasmic

reticulum with free ribosomes, large vacuolated mitochondria with dark granules, and few primary and many secondary lysosomes (Martínez-Maqueda et al. 2015). These cells consume high levels of glucose, and hence in vitro growth of these cells needs a medium containing elevated levels of glucose. They propagate as a multilayer of nonpolarized cells under standard conditions. After the treatment with inducers, these cells can be modulated to express various paths of absorptive cell differentiation, such as cell flattening, apical surfaces with brush border development, and formation of tight junctions among the adjoining cells. These intestinal cells are pluripotent and widely used to study cell differentiation mechanisms (Martínez-Maqueda et al. 2015). The p53 (a cellular tumor protein) antigen is overproduced in these cells. HT29 secretes pro-inflammatory cytokines, namely, tumor necrosis factor-α (TNF α) and interleukins (IL-1β and IL-6); chemokines (e.g., interferon-γ and IL-8); transforming growth factors (TGF-α and TGF-β); pro-angiogenic factors like vascular endothelial growth factor (VEGF) and IL-15; and immune-modulatory cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (GCSF), and IL-3 (Desai et al. 2013).

Orientin expressively inhibits the cell survival of HT29 in a concentration-dependent way. Further, microscopic interpretations suggested the cell shrinking from its polyhedral origin, membrane blebbing, round off, and cells in detached forms (Karthi et al. 2016). The oxidative stress-induced intracellular ROS (reactive oxygen species) generation in tumor cells serves to be one of the possible therapeutic strategies for combating CRC. Excessive generation of ROS may severely damage the genome and proteins, resulting to promote apoptosis (Han et al. 2013). Orientin triggers dose-dependent intracellular ROS generation extensively.

1.4.2 Chemically Induced Animal Models

Chemical carcinogens are extensively used to induce colonic epithelial lesions similar to human CRC. They include aromatic amines, 1,2-dimethylhydrazine (DMH), heterocyclic amines, azoxymethane (AOM), and alkylnitrosamide compounds. These carcinogens can be readily verified for their therapeutic effects in model animals of diverse genetic conditions, and the human CRC pathogenesis can be initiated successfully (De Robertis et al. 2011). The anticancer efficacy of chemopreventive agents is generally tested against carcinogen-induced preclinical models. The rodents (rat/mouse) are commonly established animal models of colon carcinogenesis. This is because their physiology is similar to humans. Also the cancer formation is rapid and reproducible and there is a possibility of studying adenoma-carcinoma sequences (Muthu et al. 2016).

1.4.2.1 DMH-Induced Experimental Colon Carcinogenesis

The procarcinogen 1,2-dimethylhydrazine dihydrochloride (DMH) was extensively used to stimulate colon tumors in rodents. It mimics human colon carcinoma in epithelial origin, morphology, anatomy, histology and tumor characteristics, thereby

acting as an ultimate experimental model for chemoprevention studies (Manju and Nalini 2005; Nirmala and Ramanathan 2011; Muthu and Vaiyapuri 2013). DMH induced the transformation of pre-neoplastic aberrant crypt foci into adenomas and adenocarcinomas. DMH and its inter-metabolites (AOM and MAM) are a set of man-made composites with the general structure of cycasin (Liu et al. 2015). DMH uptake is threefold higher in the colorectal cells in contrast to the enterocytes. The cancer-causing effect of DMH occurs with a single dosage of injection or by giving a series of injections after every week. DMH injected at a dose between 10 and 20 mg/kg body weight (BW) produces adenomas and adenocarcinomas of the colon in rodents. After the administration, the malignant lesion is formed from the non-dysplastic mucosa, and later it completely develops within 4 to 30 weeks. About 80% of the treated mice can develop adenocarcinomas even when a lesser dose of drugs are administered (Machado et al. 2016). Injecting a small dosage (10 mg/kg) of dimethylhydrazine will lead to colorectal cancer development in rats with a latency duration of 1–2 years (Banerjee and Quirke 1998). The preneoplastic lesions namely the aberrant crypt foci (ACF) are the earliest marker of future neoplastic development that appears after exposing DMH for 14 days (Kilari et al. 2016).

1.4.2.2 Metabolism of DMH

DMH undergoes dehydrogenation in the liver and is metabolically activated to azoxymethane (AOM) and methylazoxymethanol (MAM) (Manju and Nalini 2010). Generally, DMH do not act as carcinogenic agent. However, it is metabolically activated (including N-oxidation and hydroxylation) into DNA reactive metabolites and exhibits its tumorigenic potential (Qi et al. 2015). The highly unstable MAM with a half-life of 12 h is metabolized further to form an active electrophilic methyldiazonium glucuronide by NAD + -dependent dehydrogenase. The methyldiazonium ion which has the capability to alkylate macromolecules excretes via bile and blood to reach the colonic lumen.

Cytochrome P450 enzymes are involved in the bioactivation of procarcinogens as they interfere with the polar functional groups via hydrolysis and redox mechanisms. The phase 2 enzymes, such as glutathione S-transferase (GST), N-acetyltransferase (NAT), DT-diaphorase (DTD), UDP-glucuronosyltransferase (UGT), and sulfotransferase (SULT), detoxify upon conjugation (Beyerle et al. 2015). Cytochrome P450-dependent monooxygenases (phase 1 enzymes) carry out a reaction to introduce –OH groups to produce methyldiazonium ions, which are highly reactive in nature, and this in turn alkylates DNA bases. The strong nucleophilic methyldiazonium ions interact with nucleotide bases to yield adducts. Both N^7-methylguanine and O^6-methylguanine induce the genetic mutations and tumor formation (Megaraj et al. 2014). O^6-Methylguanine induces GC to AT transitions and k-ras protooncogene mutations in DMH-induced colorectal carcinogenesis. Phase 2 enzymes, such as GST and DTD, are known to detoxify the electrophilic intermediate compounds (Giftson et al. 2010).

1.5 Orientin Against Colon Cancer in Different Ways

1.5.1 Body Weight, Growth Rate, and Polyp's Incidence

Body weight and growth rate typically controls the carcinogenicity rate in carcinogen-induced investigational animals (Manju and Nalini 2005). Loss in body weight in addition to growth rate in DMH alone induced rats could be because of increased cancer burden, lack of appetite followed by the higher incidence of polyps driven cachexia and anorexia (Vinothkumar et al. 2014a). Orientin increases the body weight despite the transformation induced by DMH, owing to their capability to reinstate the metabolic deregulation. The plant metabolites have been proven to gain body weight by restraining the cancer-causing agent-induced tumorigenicity and diminishing the incidence of polyps (Selvam et al. 2009).

1.5.2 Lipid Peroxidation and Antioxidant Status

Antioxidant and lipid peroxidation levels are anticipated to be the notorious markers for ascertaining the peril of oxidative damage-induced tumorigenesis (Thangaraj et al. 2018; Muthu and Vaiyapuri 2013). The significant increase in circulatory thiobarbituric acid reaction substances (TBARS) by the high ROS production and membrane crumbling leads to the transformation of epithelial cells (Perše 2013). Tumors acquire favorable conditions and proliferate rapidly in DMH-exposed rats where the lipid peroxidation is decreased (Giftson et al. 2010; Ghadi et al. 2009; Vinothkumar et al. 2014b). The decreased TBARS in colon tissues might be because of the concomitant resistance and reduced vulnerability of tumor cells to the ROS scavenging action (Muthu and Vaiyapuri 2013). Antioxidants defend cells from the oxidative damages via free radical scavenging activities. The antioxidants, such as CAT and SOD, primarily scavenge the reactive oxygen species; GPx detoxifies H_2O_2 and by this means counteracts reactive oxygen species. GSH and the dependent enzymes closely associate with innate defense (Siddique et al. 2017). The increased consumption of tissue antioxidants during DMH metabolite detoxification in tumor cells leads to their decreased level in DMH-alone-exposed rats. Orientin restores the antioxidant levels to put forth their scavenging action. The free -OH groups present in orientin make it an efficient antioxidative agent against ROS prompted by DMH and thereby exhibit their inhibitory prospective against colorectal carcinogenesis.

1.5.3 Xenobiotic Metabolizing Enzymes

DMH upon dehydrogenation in the liver forms azoxymethane (AOM) and methylazoxymethanol (MAM) intermediates (Manju and Nalini 2010). Phase 1 and phase 2 enzymes introduce polar or reactive groups into xenobiotics or carcinogens. Phase 1 enzymes stimulate the procarcinogens by introducing polar active groups, while the phase 2 enzymes detoxify after conjugating with the carcinogens (Padmavathi

et al. 2006; Beyerle et al. 2015). Cytochrome P450 enzymes effectively convert lipophilic xenobiotics into more hydrophilic carcinogenic compounds (Sangeetha et al. 2012). The increased level of liver microsomal drug-metabolizing enzyme and intestinal epithelial cell phase 1 enzymes, i.e., CYP2E1, CYP450, and cytochrome b, exhibit DMH-induced carcinogenicity. The genotoxic intermediates produced by phase 1 enzymes covalently attach to form DNA adducts (Balaji et al. 2014). Similar to many flavonoids, orientin administration activates cytochrome P450 enzymes owing to the existence of –OH groups (Sangeetha et al. 2012). The phase 2 enzymes, comprising GST and DT-diaphorase, aid in the introduction of a polar or reactive group to xenobiotic compounds. DT-diaphorase, a widely distributed flavoprotein in animal tissues, detoxifies the quinone and its derivatives to protect against neoplasia (Mohan et al. 2006). Glutathione-S-transferase (GST) interrupts initiation of carcinogenesis by detoxifying hydroquinones and neutralizing electrophilic intermediates (Balaji et al. 2014). The increased level of phase 2 enzymes aids protection against carcinogens. The decreased phase 2 enzyme levels in the hepatic and colonic regions might be because of utilizing more detoxifying enzymes for counteracting DMH-induced malignant tumor formation. Orientin increased the level of phase 2 enzymes and detoxifies the carcinogens. Orientin diminishes active DMH metabolite formation and excretes carcinogen from the colonic lumen.

1.5.4 ACF Formation

Aberrant crypt foci are surrogate precursor lesions of colon cancer distinguished from the usual crypts based on their size, shape, thickness, and the pattern of staining (Bird and Good 2000; Rodrigues et al. 2002). The aberrant crypt with high multiplication rate, i.e., >4 crypts/focus, and their number are the indication of colorectal cancer incidence (Aranganathan and Nalini 2009). The carcinogen-induced rats show higher incidences of ACF and cancer frequency (Baskar et al. 2011). The bioactive compounds inhibiting ACF would also promote the antitumorigenicity (Sengottuvelan et al. 2006; Muthu and Vaiyapuri 2013). ACF shows the initiation of colorectal cancer formation and their increased numbers and multiplicity of crypt suggest the advancement and progress of cancer. Orientin inhibits the ACF progression and suppresses the transformation to malignant neoplasia. The protective activity is ascribed to the antioxidative efficiency of orientin and the metabolic activation of xenobiotic enzymes. The capability of orientin to reinstate DMH-stimulated histological changes authenticates its anticancer and antiinflammatory potentials.

1.5.5 Tumor Marker Levels

An early identification and lessening of precancerous lesions can be made possible by the quantification of serum markers, which may curtail the incidences and death rate of CRC. The assessment of tumor marker levels in serum acts as the prominent

markers owing to their ease of handling and economical. The Serological cancer markers (CEA and CA 19–9) are synthesized and liberated into the interstitial fluid and then enters lymph to enter circulation (Narimatsu et al. 2010). Cells holding a higher metastatic potential express these intracellular adhesive proteins on their surface. CEA, the glycosylated immunoglobulin, is the most often characterized tumor-associated antigen and widely existing biomarker for CRC patients. CEA is found in high levels during the malignant and metastatic stages compared to benign conditions (Shitrit et al. 2005). CEA levels in the columnar epithelial cells and goblet cells vary with tumor staging (Flamen et al. 2001). The elevated levels of CEA found in DMH-stimulated experimental animals are most probably linked with tumor size, stage, multiplication, and tumor site. Orientin is reported to moderate CEA levels via reducing the rate of tumor development. CA 19–9 is one of more familiar cancer markers for diagnosing CRC. The increased level of this glycolipid is highly correlated with increased CRC mortality cases. The increased CA 19–9 levels is highly related to perineural and lymphatic invasions, leading to metastatic tumors (Fernandez-Fernandez et al. 1995). The decline in the levels of CA 19–9 after injecting orientin hypothesizes the antitumor action, similar to that of umbelliferone (Muthu and Vaiyapuri 2013). Moreover, orientin treatment for the whole period revealed a strong influence of its cancer preventive potential in DMH-prompted colorectal cancer-possessing experimental animals.

1.5.6 Mast Cell Infiltration

Infiltration of mast cells acts as the finest marker for beginning of inflammatory process. The innate cells of the immune system alter the progression of adenomas into carcinoma initiation by eliciting inflammatory reactions (Khan et al. 2013). Mast cell infiltration is apparently observed in the submucosal layer of DMH-treated animals, which aggravates constant inflammation in the tumor microenvironment. Orientin reduced mast cell infiltration to indicate that it has a potential antiinflammatory activity and also anti-angiogenic potential. The reported antiinflammatory effect of orientin was similar to carvacrol and umbelliferone (Muthu and Vaiyapuri 2013; Arivalagan et al. 2015).

1.5.7 Tumor Cell Proliferation

The reliability of intestinal mucosa is maintained by the crucial mechanism of cell proliferation. The deregulation of cancer cell multiplication recurrently effects in hypergenesis and oncogenesis (Lee and Yun 2010). The nucleolus-associated chromosomal regions (nucleolar organizer regions) are positioned on the acrocentric chromosomes short arm. NORs containing the acidic proteins are silver-stained (AgNORs), and they serve as the investigative or predictive marker of cell multiplication/proliferation (Gundog et al. 2015). In cancerous cells, the cell proliferation is positively linked to AgNORs/nucleus (Sengottuvelan et al. 2006). The total number

of AgNORs/nucleus determines cancer succession and the developmental phases. The black dots visualized are utilized as prognostic indicators for cell spread/propagation (Arivalagan et al. 2015). The aggregated numbers of AgNORs/nucleus in the colonic epithelium were found to be higher in DMH-treated experimental animals (Sengottuvelan et al. 2006; Muthu et al. 2016). Orientin decreases the number of AgNORs/nucleus owing to its role in preventing the proliferation of cancer cells. Umbelliferone was earlier reported to decrease the number of AgNORs/nucleus in enterocytes (Muthu et al. 2016; Mariyappan et al. 2017).

Proliferating cell nuclear antigen (PCNA), a non-histone nuclear acidic protein (of 36 KDa), is presumed as an intermediate biomarker of cell proliferation (Aranganathan and Nalini 2013). The expression of PCNA was high in the nuclei of multiplying cells in the growth (G1) phase and early synthetic (S) phase of cell cycle. PCNA monomers enclose the DNA strand like a ring and hold DNA polymerase δ (DNA Pol δ) to the template strand as a clamp to cause hyperproliferation (Mohania et al. 2014). Another proliferating antigen, Ki67 expresses only during G1, S, and G2 phases, but not in the G0 phase. The augmented nuclear expression of PCNA and Ki67 in DMH-induced rats indicates a high proliferation of colonic epithelial cells (Sekar et al. 2016). The suppression of cell proliferation represents one of the protecting actions against DMH-treated colon cancer. Orientin was shown to decrease the expressions of PCNA, Ki67, and their labeling indices. Orientin inhibits cell proliferation by its anti-apoptotic activities (Karthikkumar et al. 2015).

1.5.8 NF-κB and Inflammatory Cytokine Expression

Tumors develop and promote the inflammatory signals in and around the microenvironment. NF-κB activation allows translocation of NF-κB dimer to initiate the transcription of pro-inflammatory cytokines and the downstream target genes (Umesalma and Sudhandiran 2010). The degree of inflammations in DMH-treated animals was apparent by increased NF-κB expression. Orientin reduced the expression of NF-κB owing to its antiinflammatory potentials against DMH-treated inflammations. The inflammatory cytokines (IL-6 and TNF-α) are produced in the tumor microenvironment, which regulates proliferation and apoptosis. The increased TNF-α level during chronic inflammation upholds tumor propagation and metastatic behavior (Colussi et al. 2013). IL-6 is mostly formed by macrophages and monocytes, following the activation of NF-κB. IL-6 plays a huge role during the acute inflammation, tumor cell multiplication, and apoptotic mediated cell death (Dai et al. 2014). TNF-α and IL-6 inhibition serve one of the effective approaches in the treatment of CRC. The elevated production of cytokines during DMH-induced inflammatory response gets downregulated because of the inhibitory potential of orientin (Nash and Ward 2014).

1.5.9 Pro-Inflammatory Enzymes

Inducible nitric oxide synthase (iNOS), the pro-inflammatory mediator, synthesizes nitric oxide (NO), which arbitrates inflammation and persuades tumorigenesis. The carcinogen-induced tumor-bearing rats increased iNOS expression, thereby promoting the invasiveness and metastatic potential (Muthu et al. 2016). iNOS overexpression in colonic cells associates with CRC pathogenesis, where it restrains apoptosis via nitrosylation of caspases. NO impedes DNA repair mechanisms and leads to cytokine post-translational modifications which further influence the commencement and progression of CRC (Narayanan et al. 2003). Orientin distinctly improves the expression of iNOS in DMH-induced rats.

Cyclooxygenase enzymes (COX-2) catalyze prostaglandin synthesis from arachidonic acid, which gets induced at the inflammatory phase and overexpressed in colonic adenocarcinoma (Hamiza et al. 2012a, b). The inflammatory chemokines, cytokines, and tumor promoters induce COX-2 activation (Peng et al. 2013). COX-2 enzymes express higher in cancerous cells via the activation of NF-κB mediated inflammatory cytokine pathway. The increase in COX-2 expression in DMH induced rats was due to its anti-apoptotic effect on colon cancerous cells (Srimuangwong et al. 2012). Orientin distinctly decreased COX-2 expression and corroborates their inhibitory action against inflammation-linked colon tumorigenesis. Orientin suppress the overexpression of inflammatory cytokines due to DMH treatment, thereby validating their antiinflammatory and antiproliferative effects.

1.5.10 Cell Cycle Arrest

The cell cycle dysregulation and avoidance of apoptotic mediated cell death are the familiar events in CRC development (Wu et al. 2018). Arresting of cell cycle at a particular checkpoint and apoptotic induction are the most commonly used mechanisms in the chemoprotection of tumors (Song et al. 2017). The cell cycle checkpoints reinforce the differentiating cells from DNA damages and control the genomic integrity. Orientin stimulated cell cycle arrest at G0/G1 phase in a concentration-dependent way. The stimulated cyclin-dependent serine/threonine kinases and their regulatory cyclin subunits regulate cell cycle progression. These cyclin/CDK complexes act as a biomarker for tumor cell multiplication and targets for anticancer drug development (Peyressatre et al. 2015). Among the different cyclin/CDK complexes, cyclin D and cyclin E along with CDK2 and CDK4 control mitotic division and advance the cell cycle through G1 phase. Orientin noticeably reduced the expression of cyclin D1, cyclin E, and the cyclin-dependent kinases, CDK2 and CDK4. Pelargonidin also showed the reduced expression of CDKs and cyclins in colorectal cancer cell line, HT29 (Karthi et al. 2016). Orientin elevates $p21^{WAF1/CIP1}$, the chief inhibitor of cyclin D/CDK complex. $p21^{WAF1/CIP1}$ mediates G0/G1 phase arrest in HT29 cells (Kan et al. 2013). CDK4 activated phosphorylation of Rb trigger the disruption of tumor suppressors which further discharges E2F and

initiates G1 to S phase transition (Asghar et al. 2015). Orientin reduces the expression of pRb and thereby inhibits G1 to S phase transition.

1.5.11 Apoptosis

Apoptosis is a complex sequence, which regulates cell proliferation and protects cells from being malignant by getting rid of immortal or repaired cells. Multiple signals trigger the loss of mitochondrial membrane potential with the successive release of cytochrome C from cytosol in carcinogen-induced cancer cells (Lemieszek et al. 2016). Apoptosis is conceivably the powerful defense mechanism of many chemotherapeutic agents toward CRC. The evolutionarily conserved members of Bcl-2 protein family such as Bcl-2 and Bax regulate apoptosis. The pro-apoptotic Bax confines to mitochondria and triggered cytochrome C release leading to caspase-mediated cell death. The prosurvival Bcl-2 binds along with Bax to avert its oligomerization and thwarts mitochondrial membrane depolarization and thereby cytochrome C release (Ding et al. 2010; Tanwar et al. 2010). Orientin decreased the level of anti apoptotic Bcl-2 and Bcl-XL with the increased pro apoptotic Bax and Bid levels which obviously demonstrated the immense potential of Orientin in regulation of Bcl-2 family proteins and apoptotic induction in colon tumor cells. Orientin activates mitochondria-mediated apoptosis in DMH-induced CRC-bearing rats by the simultaneous increase of cytosolic cytochrome C with a decrease in Bcl-2/Bax ratio. Orientin increases caspase 3 and caspase 9 expression in tumor-bearing rats. Caspases are the aspartate-specific cysteine proteases that play a decisive role in apoptotic process. The binding of cytosolic cytochrome C with Apaf-1 activates caspase 9, the initiator caspase, and in turn activates the caspase 3, the downstream effector which leads to intrinsic apoptosis (Sengottuvelan et al. 2009). Orientin activates mitochondria-mediated intrinsic apoptosis in DMH-induced CRC-bearing rats.

Orientin induces Smac/DIABLO release in HT29 cells, along with cytochrome C, thereby neutralizing inhibitor of apoptosis proteins (IAPs) (Srinivasula et al. 2000; Endo et al. 2009; Abdel-Magid 2017). The cytochrome C associates with apoptotic protease-activating factors (Apaf-1) and procaspase 9 to form apoptosome and commence the activation of caspase cascade. The binding of Apaf-1 triggers a conformational change in procaspase 9 to caspase 9 (Omer et al. 2017). Orientin increases caspases (caspase 9 and caspase 3) and cleavage of poly(ADP-ribose) polymerases. Orientin induces apoptosis primarily in the intrinsic pathway (Jiang et al. 2017; Li et al. 2017b). Orientin decreased the expression of inhibitor of apoptosis protein family members, XIAP and Survivin, in HT29 cells due to the release of cytochrome C and the depolarization of mitochondrial membrane potential (Abdel-Magid 2017). Orientin increased expression of tumor suppressor p53 and induced overexpression of $p21^{WAF1/CIP1}$. The increased level of γ-H2AX in Orientin treated tumor cells serves as a hallmark of DNA damages which confirms the DNA damage induced in HT29 cells.

1.6 Conclusions

Colorectal cancer is one of the most diagnosed cancers, which can be protected or prevented with the chemotherapeutic agents; however, the available drugs have shown systemic toxicity and drug resistance which makes the treatment a challenging issue. A positive correlation exists between flavonoid-rich sources and lowers the risk of colorectal cancer. The above discussed contents explored the effect of dietary flavonoid, orientin, and validated its anti-carcinogenic effect in human colorectal adenocarcinoma HT29 cells and DMH-induced colorectal cancer-bearing Wistar rats. Orientin induces mitochondria-mediated intrinsic apoptosis in colorectal cancer cells and arrests tumor growth by interrupting cell proliferation and inflammatory signaling pathways. Orientin exerts a regulatory effect on major signaling pathways linked with colon cancer progression, namely PTEN/PI3/Akt and Wnt/β-catenin pathways. Overall, this chapter suggests that orientin can be a novel chemotherapeutic agent for controlling CRC.

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