# Citrus Peel Flavonoids as Potential Cancer Prevention Agents

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## ABSTRACT

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## Medicinal Properties of Citrus Fruits

Citrus fruits such as mandarin, pomelo, orange, lime, lemon, and grapefruit have been recognized as having high contents of bioactive compounds (1). Between the pulp and the peel, such fruits contain folate, vitamin C, dietary fiber, and bioactive compounds such as flavonoids. Flavonoids are widely distributed in aromatic plants such as mint and tea but are present in high concentrations in citrus fruits and their peels (2).

Citrus peel has untapped potential as a source of medicinal compounds because it contains carotenes, essential oils, pectin, and a range of polyphenolic compounds (3). Epidemiological studies have suggested that high consumption of fruits and vegetables (>400 g/d) can reduce cancer risk by ≥20% (4). The Mediterranean diet is rich in fruit pulp and juice, and the associated high intake of fiber, antioxidants, and polyphenol compounds is linked with a lower cancer risk (5, 6).

The medicinal use of citrus peels can be traced back to the 10th century, but the biological activities of specific chemicals within the peel have only recently been characterized (7, 8). Citrus peels are rich in polyphenolic compounds, which are secondary plant metabolites with diverse and essential biological functions (9, 10).

Polyphenolic compounds consist of various classes of bioactive compounds including flavonoids, limonoids, coumarins, phenolic acids, terpenoids, tannins, stilbenes, lignans, and carotenoids (11–13). They contain heterocycles including aromatic rings with hydroxyl groups in their basic structure (14) and exist in the free state or as glycosides. Flavonoids are likely to be key bioactive compounds in citrus peel, particularly in terms of their anticancer activity (15–17) as well as in the prevention of infectious and degenerative diseases (18–20). Although it is appealing to identify specific molecules with high anticancer activity, there is growing evidence to suggest synergy between bioactive molecules in citrus peel extract (CPE). Whole CPEs have been shown to have higher anticancer activity than the fractionated extracts and isolated single compounds. Indeed, the methanolic extracts and freeze-dried CPEs are correlated with higher concentrations of total phenolic and flavonoid contents (21–23).

Several salient reviews should be noted. Cirmi et al. (4) detail the range of individual flavonoid and polyphenolic compounds found within citrus fruits and summarize the preclinical and epidemiological evidence for their utility in cancer treatment. Kandaswami et al. (24) describe

the general utility of flavonoid compounds (not specifically from citrus) in modulating cell signaling pathways. This critical review focuses on the bioactive compounds that are enriched in citrus peel and examines their underlying mechanism of action. This is timely based on growing efforts to utilize CPEs as chemopreventive agents (25), as well as to leverage their antiatherogenic, anticarcinogenic, anti-inflammatory (26), anticancer (27), antidiarrheal, and antimicrobial properties (3, 28). In this extensive field, such studies are challenging to compare due to a lack of standardized in vitro and in vivo methodologies, as well as the use of whole CPE compared with individual polyphenolics, flavonoids, flavonols, flavones, and polymethoxylated flavones. However, this review explores a range of common mechanisms that feature in preclinical studies including motivation of carcinogen detoxification, scavenging of free radical species, control of cell cycle progression, preventing the initiation of cancer, inhibiting cell proliferation, increasing apoptosis, reducing oncogene activity, prohibiting metastasis and angiogenesis, as well as modulating hormone or growth factor activity (4, 29–32). This involves highlighting both recent and historical reports and synthesizing a model for the different biological functions of CPE bioactives. In most cases there has been no proper follow-up, either in vivo or in clinical research.

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## Flavonoid Subtypes within CPE

Flavonoids are low molecular weight compounds that are responsible for the vivid color of fruit peels, pulp, and leaves (11). They are found abundantly in citrus fruits, seeds, olive oil, red wine, and tea. More than 9000 flavonoids have been identified to date. Flavonoids feature a basic $C_6$–$C_3$–$C_6$, 15-carbon skeleton. They are comprised of 2 benzene rings (A and B), which are linked via a heterocyclic pyran ring (C in **Figure 1**). Flavonoids are subdivided according to the presence of an oxy moiety at C4, a double bond between positions 2 and 3, or a hydroxyl group in position 3 of a heterocyclic ring (C in Figure 1).

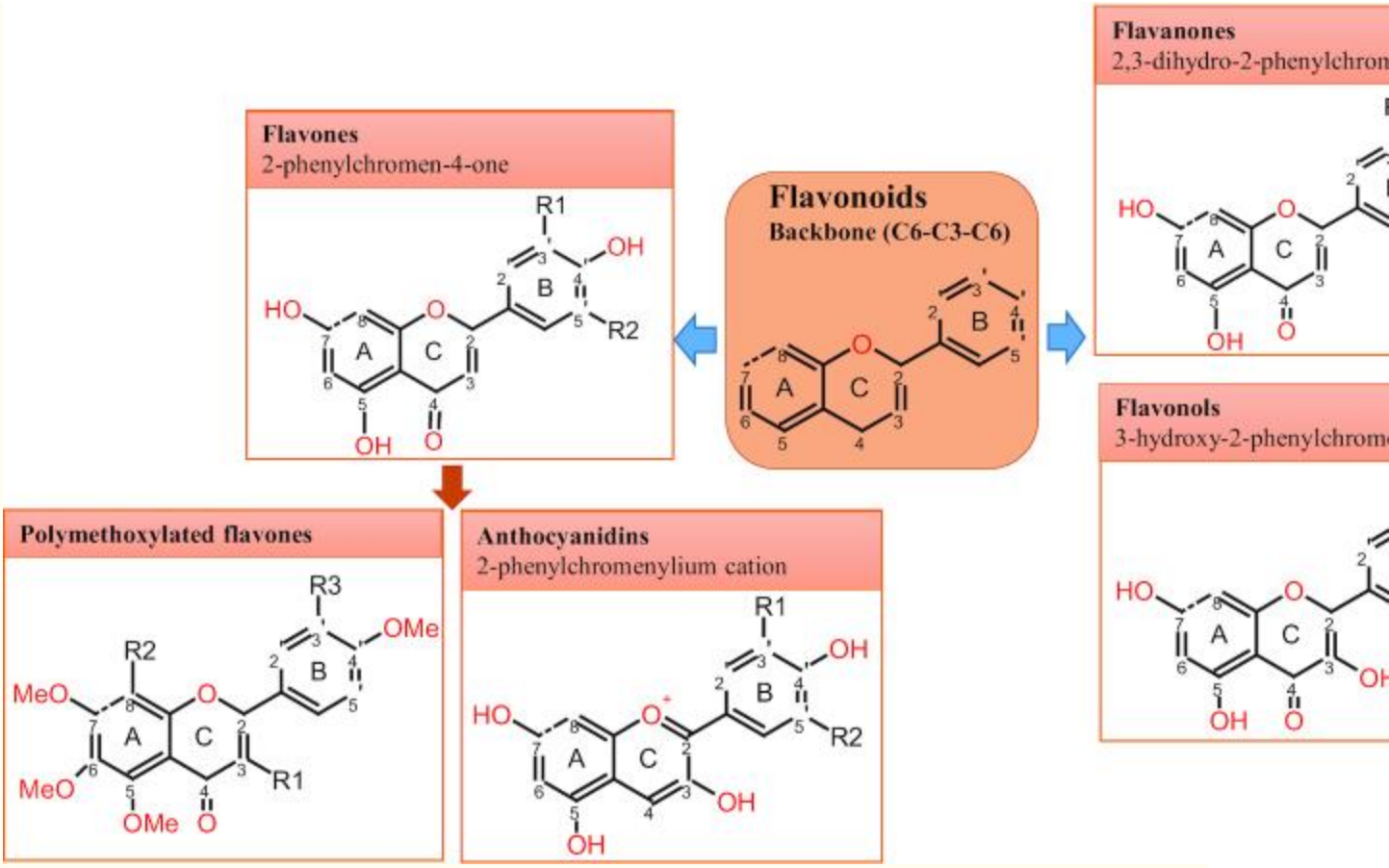


FIGURE 1

Main skeleton of flavonoids and their classes.

The biological activities of flavonoids increase with the degree of hydroxylation of the B ring (Figure 1) (24, 33). The basic structure of flavonoids permits a significant number of substitution patterns in the benzene rings A and B within each class of flavonoids: O-sugars, methoxy groups, phenolic hydroxyls, sulfates, and glucuronides (2, 34). The abundance of distinct flavonoids arises from a large number of different combinations of hydroxyl and methoxyl group substitutions. Besides, flavonoids can be classified by variations of the heterocyclic ring C to flavones, flavanones, flavonols, isoflavones, flavans, and anthocyanidins (9, 35).The antioxidant activity of flavonoids is related to *ortho*-dihydroxy substitution in ring B, the presence of a 2,3 double bond and of a 4-oxo moiety in ring C, as well as a 3-hydroxy-4-keto and/or 5-hydroxy-4-keto conformation in rings C and A (36, 37).

Flavonoids with a hydroxyl group in position C3 of the C ring are termed flavonols, and those lacking such an –OH moiety are called flavanones and flavones. **Figure 2** illustrates the main structural formulas of some flavonoids isolated from CPE and their structural variations. The main abundant flavonoids in CPE are flavanones such as neohesperidin, naringin, and hesperidin (38–42) as well as nobiletin, sinensetin, and tangeretin (43). The biological activities of flavonoids are related to their antioxidant properties (44). The different degenerative diseases such as brain diseases and Alzheimer disease are affected by flavonoids via their antioxidant properties (42, 45, 46). There is evidence linking the pharmacological activity of CPE flavonoids

to their ability to reduce the activity of intracellular signaling molecules including topoisomerases, phosphodiesterases, and kinases, as well as other regulatory enzymes (45, 47).

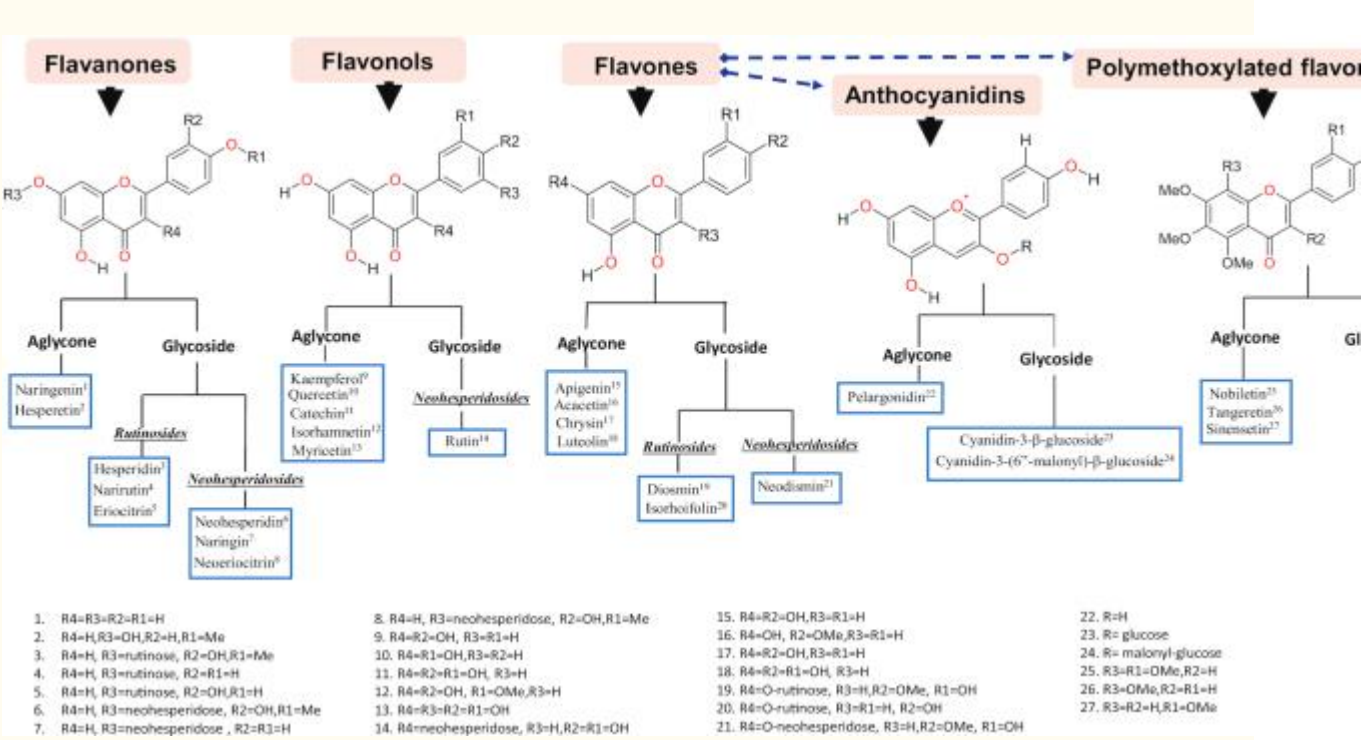


FIGURE 2

The structural formulas of the main citrus peel flavonoids and their subclasses.

Flavanones (2,3-dihydro-2-phenylchromen-4-one) are a major class of flavonoids and occur mostly in glycoside forms such as hesperidin, neohesperidin, narirutin, naringin, eriocitrin, and neoeriocitrin. The glycosidic forms are divided into 2 types—rutinosides and neohesperidosides. Both rutinose and neohesperidose are glycosylated at position 7 and disaccharides are formed by glucose (Figure 2). The bitter taste of neoeriocitrin, naringin, and neohesperidin is caused by the presence of neohesperidose (rhamnosyl-α-1,2 glucose) in flavanones. Hesperidin, narirutin, and eriocitrin consist of a flavanone bound to rutinose (rhamnosyl-α-1,6 glucose), and they have no taste. The most critical flavanones in aglycone forms are naringenin and hesperetin.

Flavonols (3-hydroxy-2-phenylchromen-4-one), such as kaempferol, quercetin, catechin, and isorhamnetin, are aglycone forms of flavonoids. Flavonols are recognized by the presence of a 2,3-double bond and the 4-oxo group in the C ring. They differ in the presence of 1 additional –OH moiety at position C3 in the C ring. Additionally, the 3-OH group can be glycosylated by different sugars, which significantly increases the number of flavonol isomers (48). The glycoside flavonols such as rutin are found in trace amounts in citrus peel. The predominant types are 3-*O*-monoglycosides, and glycosylation occurs at the 3-OH group of the C ring (4).

Flavones (2-phenylchromen-4-one) are found in low concentrations in citrus peel. Nevertheless, they can produce important biological activities in vitro and in vivo. For instance, apigenin has shown high anti-inflammatory activity, and diosmin is an important venotonic agent (49, 50). Methylated flavones are the key flavones noted in citrus fruits (51).

Anthocyanidins (2-phenylchromenylium cation) are structurally derived from pyran, flavan, and flavones found only in grapefruit and blood oranges (4). Anthocyanidins are the aglycone counterpart of anthocyanins that are natural pigments of fruits responsible for the fruits' and flowers' violet, red, and blue coloring. The color of the anthocyanin occurs in response to changes in pH, oxygen, temperature, light, and enzymes and also by methylation or acylation at the hydroxyl groups on the A and B rings (52).

Polymethoxylated flavones (PMFs) are a subdivision of flavones with ≥2 methoxyl groups on their basic benzo-γ-pyrone skeleton and a carbonyl moiety at the C4 position. Notable PMFs include tangeretin, nobiletin, and sinensetin. PMFs exist exclusively in citrus peels and have been used as herbal (alternative) medicines for decades (49, 53). In research studies, PMFs have shown a broad spectrum of biological activities including anticarcinogenic (54, 55), antioxidant, cardiovascular protection, antiproliferation, antiatherogenic (56, 57), and anti-inflammatory activities (7, 55, 58–60). The permeability of PMFs through biological membranes is higher than other flavonoids because of their planar structure and low polarity (58, 61).

The antioxidant, enzyme-inhibitory, and antiproliferative activities of flavonoids are related to their specific structural features including the presence of glycosylation, the structure oxidation state, and the substituents in both the A and B rings of the flavonoid structure (62, 63). Studies of melanoma cell lines employing several flavonoids of citrus peels have shown the presence of the C2=C3 double bond on the B ring, conjugated with the 4-oxo function, to be critical for this biological activity (64). The presence of ≥3 hydroxyl/methoxyl groups in each ring (A or B) of the flavonoid skeleton significantly increased the antiproliferative activity in human melanoma B16F10 and SK-MEL-1 cell lines (64, 65).

Up to 62 glucoside and aglycone limonoids have been reported in citrus fruits (66). Obacunone glucoside and nomilin acid glucoside are the major limonoid glucosides in CPEs (67). Coumarins are another class of bioactive compounds mainly present in citrus peel. Coumarins such as 7-methoxy-8-(2-oxo-3-methylbutyl) coumarin, 5-geranyloxy-7-methoxycoumarin, auraptene, limettin, and epoxyaurapten, as well as furanocoumarins such as psoralen, xanthotoxin, bergamottin, and epoxybergamottin have been found in citrus peels (68–71). Cinnamic acids (caffeic, *p*-coumaric, chlorgenic, ferulic, and sinapic) and benzoic acids (protocatechuic, *p*-hydroxybenzoic, and vanillic) are phenolic acids found in low concentrations in citrus peel (72, 73). Meanwhile, carotenes (β-carotene) and xanthophylls [β-cryptoxanthin, lutein, β-citraurin, violaxanthin, (9*Z*)-violaxanthin, and zeaxanthin] are the main carotenoids found mostly in citrus peel (72, 74). Apart from the above bioactive compounds, D-limonene is the primary essential oil in citrus peel (75) with anticancer activity in humans (76).

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## Extraction of Flavonoids from Citrus Waste

In order to maximize the yield of bioactive flavonoid compounds from citrus peel, several different methods for extraction have been reported in the literature (77). Recommended methods include: *1*) chemical methods such as hot water extraction (78, 79), solvent extraction (80), and alkaline extraction (81, 82); and *2*) advanced methods such as ultrasound-assisted extraction, supercritical fluid extraction (83), microwave-assisted extraction (84), and enzyme-assisted extraction. The goal is to develop processes that are rapid and economical.

Most of the pharmaceutical and food industries use solvents for the extraction of bioactive compounds from citrus. Organic solvents, such as hexane, methanol, ethanol, petroleum ether, benzene, toluene, ethyl acetate, isopropanol, and acetone have been used to extract flavonoids from citrus waste. Phenolic compounds transfer from the solids to the surrounding solvents during the extraction. The temperature and time of extraction are specific for different kinds of flavonoids. The limitations of chemical methods are the several hours needed for extraction, large volumes of solvent, and the extra cost and time to evaporate the residual solvent. In contrast, "green chemistry" has emerged as a principle for the environmentally friendly extraction of high-value compounds. Such methods can be selective, low-energy, time-saving, and produce higher yields at a reduced solvent consumption (78).

The different extraction methods used for citrus flavonoids have their own advantages and limitations. However, combined approaches could ultimately prove superior to any individual method. In general, using food-grade solvents and ultrasound-assisted extraction of flavonoids from citrus waste has a strong potential for future industrial development as an efficient and environmentally friendly process (85).

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## Mechanism of Action of CPE Flavonoids

CPEs have been reported to show anticancer activity in various cancer lines at different efficacious levels; their activity is directly related to the CPE composition and the cell line sensitivity. The following sections provide an overview of the in vitro and in vivo studies showing that CPEs have potential in reducing the risk of cancer development and progression (**Tables 1** and **2**).

### TABLE 1

In vitro anticancer effects of citrus peel extract[1]

| Sample | Compound identification | Cell lines (IC50, μg/mL) | Cell cycle arrest | Antiproliferation | Proapoptosis | Antimetastasis | Anti-inflammatory and antiangiogenesis | Reference |
|---|---|---|---|---|---|---|---|---|
| *Citrus reticulata* | D | WEHI 3B (<100) | — | — | — | — | — | 106, 107 |

| Sample | Compound identification | Cell lines (IC50, μg/mL) | Cell cycle arrest | Antiproliferation | Proapoptosis | Antimetastasis | Anti-inflammatory and antiangiogenesis | Reference |
|---|---|---|---|---|---|---|---|---|
| *C. reticulata* | — | SNU-668 (~100) | — | — | I | — | — | 108 |
| *C. sinensis* | D | MCF-7 (10.2–17.9) | — | — | I | — | — | 109 |
| *C. grandis* | D | U937 (60), HepG2 (31), HeLa (287), HCT-15 (87), MCF-7 (110), NCI-H460 (73), | — | — | I* | — | — | 68 |

| Sample | Compound identification | Cell lines (IC50, μg/mL) | Cell cycle arrest | Antiproliferation | Proapoptosis | Antimetastasis | Anti-inflammatory and antiangiogenesis | Reference |
|---|---|---|---|---|---|---|---|---|
| | | SNU-16 (90) | | | | | | |
| 17 citrus varieties | D | HT-29 (31–45) | — | — | — | — | — | 110 |
| *C. sunki* | D | HL-60 (25) | G2/M | — | I | — | — | 53 |
| *C. aurantium* | D | AGS (40–60) | G2/M | I | I | — | — | 38 |
| *C. aurantium* | — | U937 (40–60) | — | — | I | I | — | 111 |
| *C. grandis* | D | HeLa (100–200), AGS | — | — | I | — | — | 70 |

| Sample | Compound identification | Cell lines (IC50, μg/mL) | Cell cycle arrest | Antiproliferation | Proapoptosis | Antimetastasis | Anti-inflammatory and antiangiogenesis | Reference |
|---|---|---|---|---|---|---|---|---|
| | | (200–400) | | | | | | |
| *C. aurantium* | D | A549 (230) | G2/M | I | I | — | — | 39 |
| *C. unshiu* | — | MDA-MB-231(>200) | — | — | — | I | — | 112 |
| *C. junos* | — | HT-29 (>1200) | — | — | — | — | I | 113 |
| *C. aurantifolia* | — | MCF-7 (59) | G2/M | — | I | — | — | 114 |
| *C. aurantium* | D | A549 | — | — | I | I | — | 40 |

| Sample | Compound identification | Cell lines (IC50, μg/mL) | Cell cycle arrest | Antiproliferation | Proapoptosis | Antimetastasis | Anti-inflammatory and antiangiogenesis | Reference |
|---|---|---|---|---|---|---|---|---|
| *C. hassaku* | D | MDA-MB-231 | — | — | — | I | — | 113 |
| *C. reticulata* | D | HepG2 (20–40), HL-60 (25–50), MDA-MB-231 (25–50) | — | — | — | — | — | 42 |
| *C. paradisi*, *C. sinensis*, *C. maxima* | D | Caco-2, LoVo, LoVo/ADR | — | — | — | — | — | 115 |
| *C. hassaku* | D | SNU-1 (<25) | G1 | — | I | — | — | 116 |

| Sample | Compound identification | Cell lines (IC50, μg/mL) | Cell cycle arrest | Antiproliferation | Proapoptosis | Antimetastasis | Anti-inflammatory and antiangiogenesis | Reference |
|---|---|---|---|---|---|---|---|---|
| *C. paradisi* | | Kasumi-1 (2000) | — | — | I | — | — | 117 |
| *C. reticulata* | D | SKOV3 (~100) | — | — | I | I | | 118 |
| *C. platymamma* | D | A549 (364) | G2/M | I | I | — | I | 86 |
| *C. sphaerocarpa* | D | MDA-MB-231 (>200) | — | — | — | I | I | 113 |
| *C. iyo* | D | U266 (>400), K562 (200–400), DU145 (>400), | — | I^ | I^ | I^ | I^ | 75 |

| Sample | Compound identification | Cell lines (IC50, μg/mL) | Cell cycle arrest | Antiproliferation | Proapoptosis | Antimetastasis | Anti-inflammatory and antiangiogenesis | Reference |
|---|---|---|---|---|---|---|---|---|
| | | MDA-MB-231 (>400), HepG2 (200–400), RWPE-1 (>400) | | | | | | |
| *C. platymamma* | D | Hep3B (100–200), HepG2 (300–400) | G2/M | I[#] | I[#] | I[#] | — | 119 |
| *C. sinensis* | D | HepG2 (>500) | G1 | I | I | — | — | 120 |
| *C. reticulata* | — | HCT116 | — | — | — | — | — | 121 |

[1]D, determined; I, induced; *only for U937; ^only for DU145; [#]only for Hep3B.

## TABLE 2

In vivo anticancer effects of citrus peel extract[1]

| Sample | Animal models | Dose (route) | Duration | Effects | Reference |
|---|---|---|---|---|---|
| *Citrus junos* | HT-29 cells implanted mice | 100 mg/kg/d (i.p.) | 4 wk | Reduced tumor size, disease activity index and colon shortening | 113 |
| *C. aurantium* | A549 cells injected in mice tail vein | Twice weekly (i.p.) | 5 wk | Reduced cancer metastasis | 40 |
| *C. reticulata* | Treated leukemic cells injected into mice | — | 2/10 wk | Reduced number of tumor cells and increased mice survival time | 106 |
| *C. sinensis* | AOM-induced carcinogenesis in mice | 0.2% in diet | 26 wk | Reduced number and size of ACF, tumor burden, and incidence | 128 |
| *C. sinensis* | Western diet inducing cancer | 0.25%/0.5% in diet | 9 wk | Reduced tumor number, multiplicity, and induced apoptosis | 129 |

| Sample | Animal models | Dose (route) | Duration | Effects | Reference |
|---|---|---|---|---|---|
| Multiple citrus | DMBA-induced carcinogenesis in mice | 100/200 μL twice weekly (cream application) | 20 wk | Reduced epidermal thickness, number of papillomas, tumor incidence, and tumor weight | 127 |
| *C. unshiu* | Double-TPA application to ICR mouse skin | 8.1 nmol/30 min | 24 h | Inhibit NO and $O_2^-$ generation | 56 |
| Multiple citrus | PC-3 cells implanted in mice | 1/2 mg/kg 5 d/wk (i.p.) and 2 or 4 mg/kg 5 d/wk (o.p.) | 3 wk | Suppressed tumor size | 126 |
| Multiple citrus | AOM-induced carcinogenesis in mice | 100/200 μL 5 d/wk (o.p.) | 6 wk | Reduced number of ACF | 126 |
| *C. iyo* | DU145 cells implanted in mice | 50/200 mg/kg thrice weekly (i.p.) | 4 wk | Suppressed tumor growth | 75 |

| Sample | Animal models | Dose (route) | Duration | Effects | Reference |
|---|---|---|---|---|---|
| *C. depressa* | TEWL and epidermal thickness in UVB-irradiated mouse skin | 100 µL of 10%/d | 1 wk | Reduce photoaging in mice | 130 |
| *C. sinensis* | HepG2 cells implanted in mice | 1/10 mg/kg thrice weekly in diet | 3 wk | Reduced tumor growth | 120 |
| *C. sinensis* | AOM-induced carcinogenesis in mice | 0.01/0.05% in diet | 4/18 wk | Reduced number of ACF | 125 |

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[1]ACF, aberrant crypt foci; AOM, azoxymethane; DMBA, 7,12-dimethylbenz(α)anthracene; ICR, Institute of Cancer Research; i.p., intraperitoneal injection; o.p., oral injection; TEWL, transepidermal water loss; TPA, tissue plasminogen activator.

The following section examines the anticancer effects of CPEs reported in in vitro experiments and animal studies that elucidate the specific mechanisms involved. The anticancer effect of CPEs can be exhibited through suppression of proliferation, cell cycle inhibition, and induction of apoptosis.

## Suppression of proliferation

Cancer cells differ from normal cells by their ability to proliferate without control, resistance to apoptosis, ability to form new blood vessels, and metastasis to distant parts of the body. Flavonoids found in CPEs have been shown to suppress these events through modulation of multiple cellular proteins that inhibit cell proliferation by downregulation of oncoproteins.

In human lung carcinoma A549 cells, the methanol extract of Korean *Citrus aurantium* fruit peel inhibited cell proliferation dose dependently and also induced apoptosis (86). Similar inhibitory effects were also observed with flavonoids isolated from Korean *C. aurantium* peel in A549 cancer cells (39).

Quercetin—the aglycone form of polyhydroxylated flavonoids (flavonols) found in onions, berries, grapes, green vegetables, and apple—is one of the most highly studied flavonoids in terms of its effects on cell proliferation. It exhibits growth inhibitory effects against a range of cancer cell lines including immortal human HeLa cells (36), human epidermoid carcinoma

(A431), NK/LY ascites tumor cells, gastric cancer cells including NUGC-2, HGC-27, MKN-28, and MKN-7 (39), colon (COLO 320 DM) (39, 87), human breast (87, 88), human squamous, gliosarcoma (89, 90), ovarian (91), human pancreatic, and human liver (HepG2) cancer cells (88, 92). Indeed, quercetin's strong antiproliferative effect might be attributable to inhibition of the protein kinase C (PKC) pathway (93, 94).

Polymethoxylated flavones such as nobiletin, tangeretin, quercetin, and sinensetin showed antiproliferative activity against human lung carcinoma cells (A549), squamous cell carcinoma (HBT43) (90), gastric cancer, leukemia (HL-60), T-cell leukemia (CCRF-HSB-2), and B16 melanoma cells (95). The antiproliferative effect of naringin is correlated with the inhibition of cell survival by binding ATP on a phosphoinositide 3-kinase (PI3K) binding site; prohibition of cell growth and modulation of cell cycle–associated proteins by inhibition of the extracellular signal regulated kinase (ERK)-signaling pathway (96); and/or binding to p21 to increase the cells' nuclear antigens and block DNA synthesis (97). Naringenin and hesperetin exhibited strong antiproliferative activity against a broad spectrum of human [estrogen receptor positive ($ER^-$)] MDA-MB-435 and ($ER^+$) MCF-7 breast cancer cells, prostate (DU-145), melanoma (SK-MEL5), lung (DMS-114), and colon (HT-29) cancer cell lines (60, 90, 98–100).

Nobiletin, a major polymethoxyflavone, also enhances the cytostatic effect in ($ER^+$) MCF-7 breast cancer cells, via upregulation of inhibitors selective for the cytochrome P450 family members CYP1B1 and CYP1A1 (the main oxidizing enzymes which are major determinants of resistance) (101). Moreover, nobiletin has effectively inhibited the proliferation of human endothelial cells of human breast, prostate, skin, and colon carcinoma cells (95, 102); decreased azoxymethane (AOM)-induced cell proliferation in colonic adenocarcinoma cells (103, 104), and exhibited direct cytotoxicity in MKN-45, TMK-1, MKN-74, and KATO-III gastric cancer cells through cell cycle deregulation (105).

Cell cycle dysfunction is correlated with cancer development. Cell cycle progression is a complex and highly regulated process and consists of 4 phases: G1, S, G2, and M (122). The progression of cells from one phase to another is controlled by the coordinated interaction of cyclin-dependent kinases (CDKs) and their cyclin subunits to form active complexes. The formation of an active complex is regulated by CDK inhibitors. In normal cells, cell cycle progression is arrested when faulty DNA needs to be repaired, or further cell replication is not required. In the context of cancer, by arresting the cell cycle progression of malignant cells the tumor or metastatic cancer burden can be reduced or eliminated (123, 124).

CPEs can modulate proteins involved with cell growth such as epidermal growth factor receptor and reticular activating system (Ras), which have a range of downstream pathways including mitogen-activated protein kinases (MAPKs), serine specific protein kinase (Akt), 3-kinase PI3K/Akt, and mechanistic target of rapamycin (mTOR). Methanol extract from freeze-dried Korean *C. platymamma* flavonoids reduced the proliferation of Hep3B cells by inhibiting PI3K and Akt phosphorylation and increased the ERK1/2, c-Jun N-terminal kinase, and p38 MAPK phosphorylation; these reduced PI3K/AKT signaling and increased MAPK activity (119). Methanol extract of the peel of *C. aurantium* also suppressed the phosphorylation of Akt in U937 cells (111), and mTOR in SNU-1 cancer cell lines (116). In A549 cells, the ethanolic extract from *C. aurantifolia* peels inhibited cell proliferation dose dependently while inducing apoptosis (39, 86, 114). The suppression of growth signals was ascribed to Akt, Ras, ERK1/2, and E-cadherin in colon tumor-bearing mice (125). The treated mice showed low concentrations of

inactive glycogen synthase kinase-3β and low accumulation in cell nuclei of β-catenin, which limits the activity of signaling pathways. The oral administration of CPEs from Gold Lotion has been reported to considerably reduce the enzyme ornithine decarboxylase, which controls cell growth and proliferation through the biosynthesis and metabolism of polyamines in treated mice with colorectal cancer (125–127).

## Cell cycle inhibition

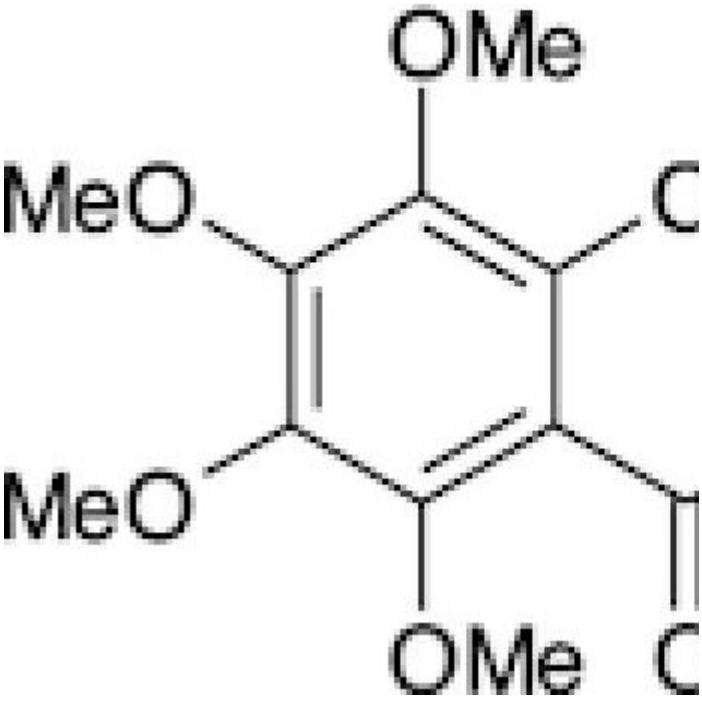
CPEs suppress cancer cell proliferation by arresting cell cycle progression and modulating cell proliferation signaling pathways that can be reduced or eliminated in malignant cells. Analysis of cell cycle distribution in CPE-treated cells demonstrated that auraptene, the main compound of the supercritical fluid extraction of *C. hassaku* Hort ex. Tanaka peel, caused cell cycle arrest mainly at G1 phase (116, 120). The ethanoic extract of *C. aurantifolia* lime peels at a concentration of 6 μg/mL induced apoptosis and cell accumulation at G1 phase, whereas the 15-μg/mL extract induced apoptosis and cell accumulation at G2/M phase (38, 39, 86, 119, 114). CPEs have been shown to upregulate the expression of p21 (cyclin-dependent kinase inhibitor 1) and/or p53 (tumor suppressor protein) leading to G1 arrest as observed in breast cancer cell line MCF-7 (114), human gastric cells SNU-1 (116), DU145 prostate cancer cells (75), and COLO 205 human colon carcinoma cells (114, 131). The CPEs can also arrest cell cycle at G2/M by increasing the expression of p21 and decreasing the expression of cyclin B1, cell division cycle 25C (CDC25C), and CDC2 in A549, Hep3B, and human gastric cancer AGS cells (38, 39, 86, 119). A water-based extract from *C. sinensis* L. peel (which chiefly contains hesperidin and narirutin) modulated the cell cycle of quiescent (PC-3 and LNCaP) prostate cancer cells, impairing their ability to enter the S phase (2–3% reduction of G0/G1 cells compared with 12–18% reduction of control cells) (132).

Tangeretin induced G1 phase by increasing the expression of p37 and p21 in COLO 205 human colon carcinoma cells (131) and prohibited the growth of estradiol-stimulated T47D cells (133). Nobiletin modulated the cell cycle in MKN-45, TMK-1, and KATO-III human gastric carcinoma cells (105), and MKN-74, and induced G1 phase arrest in MCF-7 and MDA-MB-435 breast cancer cells, and HT-29 colon cancer cell lines (134, 135). Hesperetin decreased the activity of MCF-7 breast cancer cells by accumulating cells in G1 phase through the inhibition of CDK4, CDK2, and cyclin D, upregulation of p21 and p27, and increased binding of p21 and CDK4 (136). Both tangeretin and nobiletin led to the accumulation of cells in the G1/S cell cycle in human colon and breast cancer cells. Naringin induced G1 arrest by upregulation of p21 (96). Apigenin also arrested cell cycle in G2/M phase in both androgen-insensitive PC-3 and androgen-sensitive LNCaP human prostate cancer cell lines by activation of a cyclin kinase suppressor WAF1/p21 (137) (**Table 3**).

### TABLE 3

Mechanisms and chemopreventive effects of citrus peel extract flavonoids on cancer cell lines[1]

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) | Cell cycle regulation | Arrested cell cycle progression at G1 | MDA-MB-435, MCF-7, HT-29, KATO-III, TMK-1, A549, MKN-45, MKN-74 | 39, 68, 142,130, 170–172 |
| | Antiangiogenesis, anti-inflammatory, antimetastasis | Inhibited the activity of extracellular signal regulated kinases 1/2 (ERK1/2) phosphorylation and c-JNK and | MDA-MB-435, MCF-7, HT-29 | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | | activation of the caspase pathway | | |
|  | Co-chemotherapeutic | Increased cytotoxicity of doxorubicin | MCF-7, T47D | |
| | Suppression of | Inhibited the activity of CYP1A2 | MCF-7, T47D | |

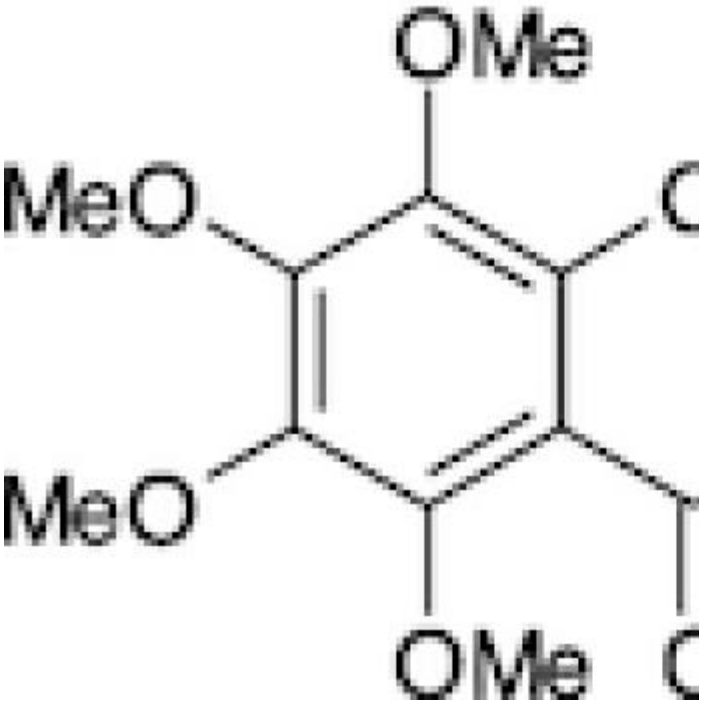
| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | carcinogenesis | | | |
| | Antioxidant | Scavenge DPPH radicals, hydrogen peroxide scavenging, hydroxyl radical scavenging | — | |
| | Antimetastasis | Prevented the migration of A549 cancer cells | A549 cells in vitro/in vivo | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Apoptosis | Downregulated (Bcl-2)/upregulation (Bax) | HeLa, THP-1 | |
| | Anti-inflammation | Decreased activation of AP-1, NF-κB, and CREB | RAW 264.7 monocyte/macrophage-like cells | |
| | Anti-inflammation | Prohibited the LPS-induced mRNA and protein expression of iNOS | Skin inflammation | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Anti-inflammation | Induced the expression of COX-2 by suppressing UVB | Human keratinocytes in vitro | |
| | Antimetastatic | Inhibited MEK1/2 activity is associated with the suppression of pro-MMPs | Human fibrosarcoma HT-1080 cells | |
| | Antimetastatic | Enhanced the expression of TIMP-1 | Human fibrosarco | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | | by the activation of PKCβII/epsilon-JNK pathway | ma HT-1080 cells | |
| | Antiproliferation | Decreased differentiation into granulocytes and macrophages by TNF-α | Murine myeloid leukemia WEHI 3B cells | |
| Tangeretin (4′,5,6,7,8-pentamethoxyflavone) | Antioxidant | Scavenge DPPH radicals, hydrogen | — | 111, 133, 136, 170, 173–177 |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | | peroxide scavenging, hydroxyl radical scavenging | | |
| | Antioxidant | Inhibited the activity of CYP1A1 and the expression of mRNA | Human intestine Caco-2 cells | — |

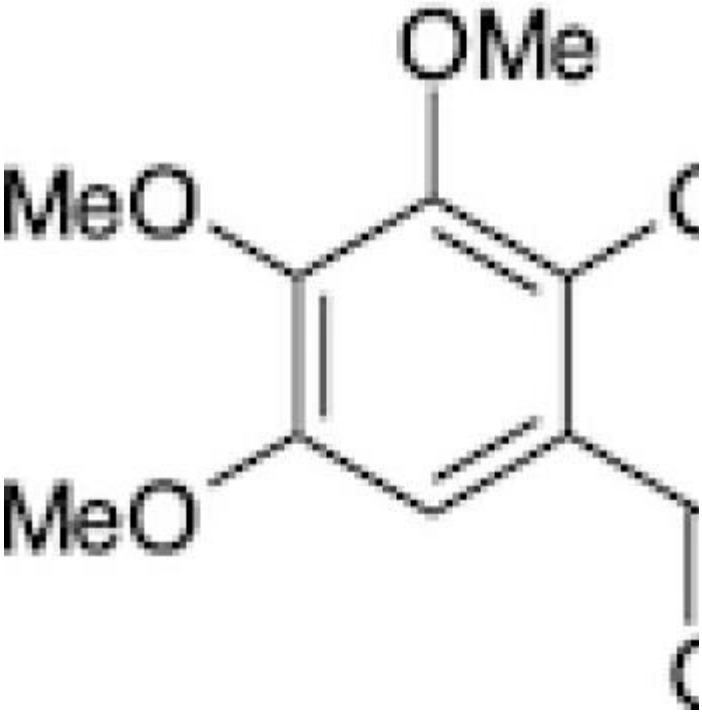
| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
|  | Apoptosis | Triggered apoptosis via p53 pathway | COLO 205, HL-60 cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Antiproliferation | Decreased the expression of PROM1 and SNAI1 | Cancer stem cell of HT29 | |
| | Antiproliferation, apoptosis | Activated caspase-3 | Cocon LOvo/DX cells | |
| | Co-chemotherapeutic | Increased cytotoxicity of doxorubicin | MCF-7, T47D | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Cell cycle regulation | Arrested cell cycle at G1 by targeting p53, p21, and p37 pathway | MCF-7, MDA-MB-435, colon cancer line HT-29, upregulate COLO 205 cells | |
| | Anti-inflammation | Blocked AKT activation | Lung carcinoma cells | |
| | Anticarcinogenic | Inhibited P450 1A/1A2/3A4 | Human liver microsome cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Antimetastatic | Decreased the number of metastatic nodules in Lentini model | Melanoma B16F10 cells | |
| | Anticarcinogenic | Reduced PhIP-DNA adduct formation in colon | Colon cancer cells | |
| | Anti-inflammation | Induced LPS-induced NO production | RAW 264.7 cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Anti-inflammation | Inhibited IL-1β-induced production of COX-2 by the activation of JNK, AKT, ERK, and p38 MAPK | A549, H1299 | |
| Sinensetin (5,6,7,3′,4′-pentamethoxyflavone) | Cell cycle arrest | Induced cells in G0/G1 phase | HUVEC | 149, 178–186 |

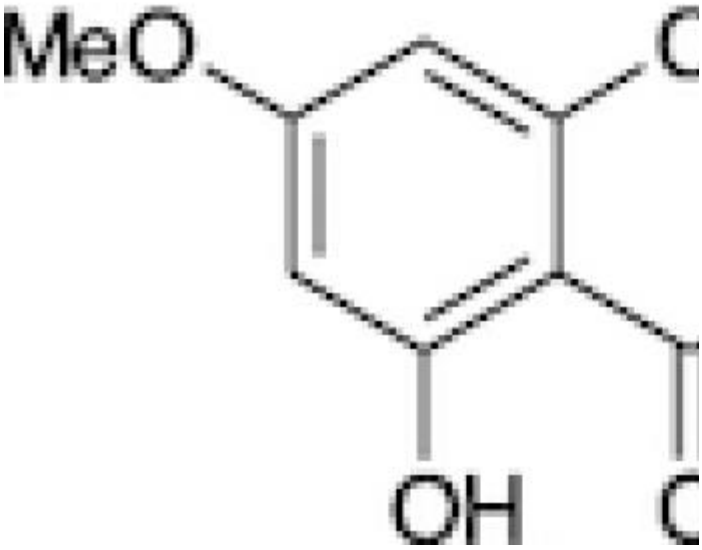
| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
|  | Antiangiogenesis | Downregulated the mRNA expression of angiogenesis flt1, hras, and kdrl | Zebrafish | |
| | Antiproliferation, apoptosis | Inhibited iNOS expression, NO production, and $PGE_2$ production | — | |
| | Cell cycle | Inhibited in S phase by | T47D breast | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | regulation | DNA elongation | cancer cells | |
| | Antiproliferation, cell cycle block | Captured cells G2/M phase and increased apoptosis, increased the expression of p53 and p21 | AGS gastric cancer cells | |
| | Anti-inflammatory | Inhibited inflammatory gene expression and STAT1 activation, | Carrageenan-induced paw inflamma | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | | inhibited iNOS, NO, and $PGE_2$ production | tion in the rat | |
| | Apoptosis | Reactivated oxygen species production, DNA damage, gene 153 expression, caspase activation | Leukemia cells | — |
| | Antiproliferation | Activated $Ca^{2+}$-dependent | MCF-7 breast | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | | apoptotic proteases | cancer cells | |
| | Apoptosis | Upregulated caspase-3, -8, -9, and poly(ADP-ribose), polymerase (PARP) cleavage | T-cell lymphoma Jurkat cells | |
| | Induced autophagy and cell death | Activated reactive oxygen species/c-Jun N-terminal kinase | T-cell lymphoma Jurkat cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | | (JNK), blocked Akt/mTOR | | |
| | Cell cycle arrest | Arrest cells at G0/G1 population | HepG2 cells | |
| | Apoptosis | Downregulated Bcl-xL, upregulated TRAIL and PTEN | HepG2 cells | |
| Hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) | Apoptosis | Induced apoptosis by activation | HL-60 cells | 39, 86, 187–193 |

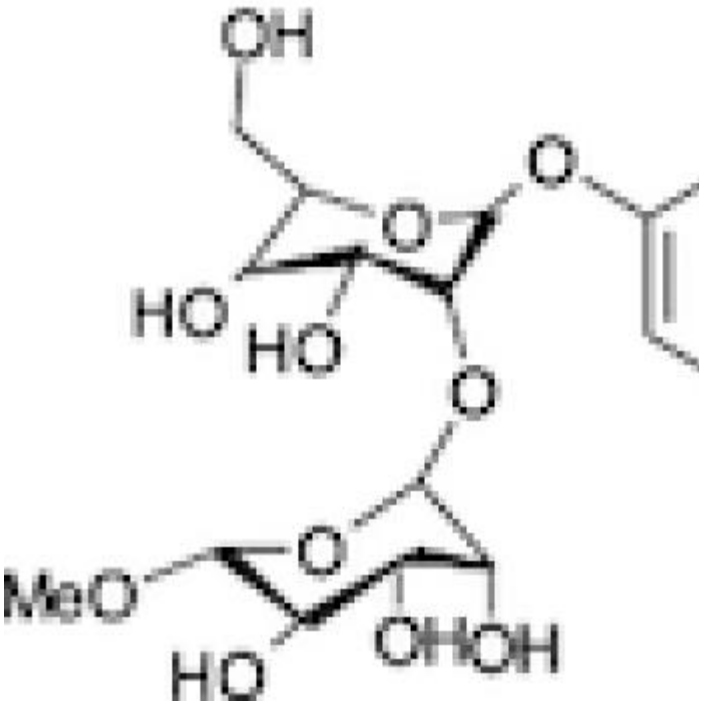
| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | | of caspase-3 | | |
|  | Antiproliferation | Inhibited oxidative stress and DNA damage | HT-29 colon adenocarcinoma | |
| | Anticarcinogenic | Downregulated the HIF-1a/VEGF/VEGFR2 pathway | Xenograft C6 glioma cells in rats | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Cell cycle arrest | Decreased cyclin D1, CDK4 and Bcl-xL by upregulating the level of p57Kip2 | MCF-7 cancer cells | |
| | Antimetastatic | Induced COX-2, MMP-2, and MMP-9 | DMH-induced colon cancer in rat; B16-F10 murine melanoma cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Apoptosis | Activated the mitochondrial pathway by rising concentrations of ROS, $Ca^{2+}$, and ATP in mice | Xenograft tumors in mouse model of gastric cancer | |
| | Apoptosis, antiproliferation | Suppressed the expression of NF-κB, p38, and caspase-3 | PC-3 prostate cancer cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Cell cycle arrest | G2/M arrest by controlling the concentration of cyclin B1, CDC2, CDC25C, and p21 | A549 lung cancer, MCF-7 | |
| | Apoptosis | Increased the expression of caspase-3, -8, -9, p53, Bax, and Fas death receptor | Cervical cancer SiHa, A549 lung cancer, HL-60 cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Apoptosis | Induced via Bax-dependent mitochondrial pathway | HT-29 cells | |
| Naringin (4′,5,7-trihydroxyflavanone-7-rhamnoglucoside) | Cell cycle regulation | Upregulated p21, G1-phase arrest, activated Ras/Raf/ERK-mediated, decreased cyclin D1 and cyclin E | 5637 bladder cancer cells, MDA-MB-231 xenograft mice | 96, 194–200 |

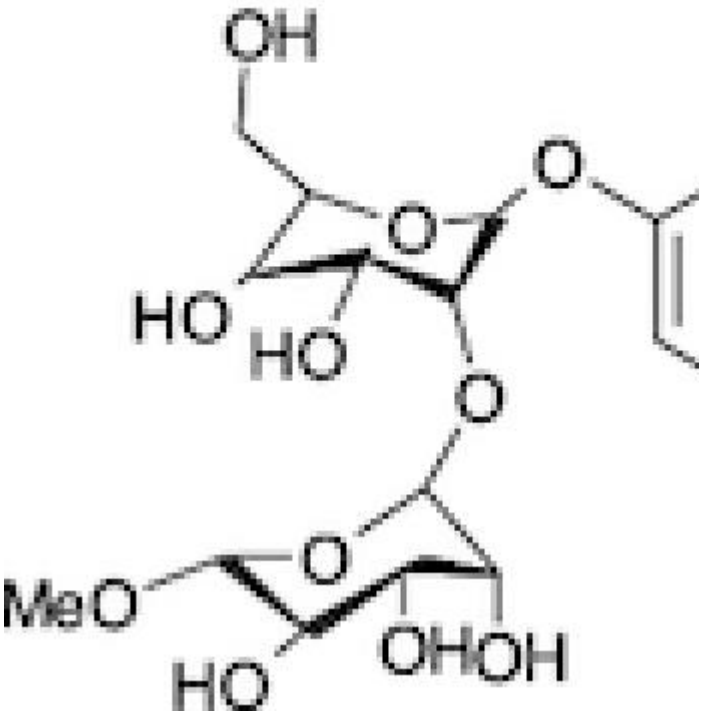
| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
|  | Metastasis, anticarcinogenic | Inhibited the activity of PI3K/Akt/mTOR and upregulated p21CIP1/WAFI | AGS cells | |
| | Cell cycle arrest | Cell cycle arrest in S phase | HT-29 | |
| | Antiproliferatio | Modulated gene | SKOV3 ovarian | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | n, antioxidant | expression, decreased DNA methyltransferase activity, downregulated the expression of Bcl2 and Bcl-xL | cancer cells | |
| | Cell cycle arrest | Increasing p21 and arrest in G1 of cell cycle; inhibited the activity of CDK2 | MCF-7 | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Antiproliferative | Inhibited CYP3A4, CYP1A2, CYP2C9, CYP2C19, and CYP2D6 | | |
| | Antiproliferation, apoptosis | Decreased the mRNA expression of BID, BAX, caspase-3, cytochrome *c*, p53, p21, and p27 | DU145 prostate cancer cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Apoptosis | Enhanced the expression of caspases, p53, Bax, and Fas death receptor | HT-29 | |
| | Antimetastasis | Downregulation of MMP-9 and repressed the PI3K/AKT/mTOR/p70S6K signaling pathway | MCF-7 | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Antiproliferation | Upregulated EGFR and ERK phosphorylation | HeLa and A549 cells | |
| | Antiproliferation, apoptosis | Suppressed the NF-κB/COX-2/caspase 1 | HeLa | |
| Hesperidin (hesperetin-7-rutinoside) | Antiproliferative | Inhibited MMP-9 by NF-κB and AP-1 signaling | NALM-6 leukemia cells | 187, 188, 201–206 |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
|  | Apoptosis | Inhibited the PI3K/Akt pathway through PTEN-phosphatase | SUN-C4 colon cancer cells | |
| | Antimetastatic, angiogenesis | Suppressing ANGPT1 gene | Laryngeal cancer cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | | Upregulated the level of p21 and p53 | MCF-7 cells | |
| | Antiproliferation | Inhibition of JAK/STAT signaling pathway | Cutaneous skin cancer cells | |
| | Apoptosis | Inhibited Aurora-A and Akt-mediated GSK-3β/β catenin cascade | A431 skin cancer cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | Antioxidant | Upregulated Nrf2 (nuclear factor-2) | Cutaneous skin cancer cells | |
| | Anti-inflammation | Downregulated mRNA expression of various cytokines (TNF, IL-1, IL-6) | Cutaneous skin cancer cells | |
| | Anti-inflammation | Inhibited IL-6, TNF, COX-2, iNOS inflammatory | A431 skin cancer cells | |

| Flavonoids | Chemopreventive and anti-inflammatory effects | Mechanisms | Cancer cells | References |
|---|---|---|---|---|
| | | components | | |
| | Antiproliferation | Upregulated BAX and downregulated Bcl-2, decreased the release of cytochrome *c* | HeLa cervical cancer cells, A2780 ovarian cancer cells | |
| | Co-chemotherapeutic | Inhibited PgP activity | Human leukemia cells (CEM/ADR5000) | |

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[1]Akt, serine specific protein kinase; ANGPT1, angiopoietin 1; AP-1, activator protein 1; Bax, Bcl2-associated X protein; Bcl, B-cell lyphoma; Bcl-xL, Bcl2-associated extra large protein; BID, a proapoptotic protein; CDK, cyclin-

dependent kinase; COX, cyclooxygenase; CREB, c-AMP response element binding protein; CYP, cytochrome P450; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EGFR, epidermal growth factor receptor; ERK, extracellular signal regulated kinase; Fas, a receptor protein of the TNF receptor family; flt, vascular endothelial growth factor receptor 1; GSK, glycogen synthase kinase; HIF, hypoxia inducible factor; hras, transforming protein p21; HUVEC, human umbilical vein endothelial cell; iNOS, inducible nitric oxide synthase; JAK, Janus-like kinase; JNK, c-Jun N-terminal kinase; kdrl, vascular endothelial growth factor receptor kdr-like; Kip2, cyclin-dependent kinase inhibitor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; mTOR, mechanistic target of rapamycin; PgP permeability glyoprotein; PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PROM, prominin-1; PTEN, phosphatase and tensin homolog; p21CIP1/WAFI, cyclin-dependent kinase inhibitor 1; P450, cytochrome P450; Raf, a serine/threonine-specific protein kinase; Ras, reticular activating system; ROS, reactive oxygen species; SNAI, sodium-coupled neutral amino acid transporter 1; STAT, signal transducer and activator of transcription; TIMP, tissue inhibitor of metalloproteinases; TRAIL, TNF-related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

## Induction of apoptosis

Apoptosis and necrosis are 2 distinct mechanisms of cell death in eukaryote cells. Apoptosis, or programmed cell death, is involved in embryonic development, hormone-dependent atrophy, and metamorphosis. These processes eliminate damaged or unwanted cells (138). The apoptosis is characterized by plasma blebbing, cell shrinkage, and fragmented nuclei/DNA (139), which are reported in a variety of cancer cells treated with CPE in vitro (38, 40, 42, 53, 68, 70, 75, 111, 116, 119, 117) and in an in vivo mouse model (140).

Citrus peel polymethoxyflavones and CPE from *C. unshiu* induce apoptosis mainly through the intrinsic pathway by reducing antiapoptotic B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra large (Bcl-XL) proteins and increasing proapoptotic proteins [Bcl-2 associated X protein (Bax); proapoptotic Bcl-2 protein (Bid); Bcl-2 homologous antagonist killer (Bak); Bcl-2-associated death promoter (Bad)] in different cancer cell lines (105, 141–143). The increase in the ratios of Bax/Bcl-XL and Bax/Bcl-2 allows the release of cytochrome *c* through the permeabilized mitochondrial membrane. Following the binding of cytochrome *c* to the apoptosis protease–activating factor 1 and formation of an apoptosome complex, activation of caspase-9 and the apoptosis effector protein caspase-3 is achieved (144).

Increase of caspase-9 and caspase-3 was reported following treatment with CPEs (super critical extract of *C. hassaku* peels) for many cancer lines including gastric carcinoma SNU-668 (108) and SNU-1 (116), adenocarcinoma human alveolar basal epithelial cells A549 (40, 86), histiocytic lymphoma U937 (68, 111), metastatic prostate cancer DU145 (75), human gastric cancer AGS (38), hepatocellular carcinoma Hep3B (119) and HepG2 (120), as well as acute myeloblastic leukemia Kasumi-1 (*Citrus* × *paradisi* Macfad.) (125, 117). CPEs increased the concentrations of cleaved poly ADP-ribose polymerase inhibitors in U937, SNU-1, AGS, Kasumi-1, A549, Hep3B, DU145, and colon cancer cells (38, 68, 75, 86,111, 116, 119, 125, 117). CPE can also reduce endogenous inhibitor of apoptosis (IAP) proteins such as XIAP, cIAP1, and cIAP2 in U937 (111) and DU145 cancer cells (75).

It was reported that nobiletin could induce apoptosis by increasing Bax and p53 protein expression, inhibiting Bcl-2 protein expression, and elevating the ratio of Bax/Bcl-2 proteins in human lung A549 adenocarcinoma cells (145). Tangeretin induced apoptosis in leukemia HL-60 cells by affecting the mitogen-stimulated blastogenic response of human peripheral blood

mononuclear cells (99), and quercetin promoted apoptosis as a consequence of cell cycle arrest in triple-negative breast cancer cells (88, 92, 146).

Accumulated evidence supports that CPE has negligible apoptosis-inducing effects through the extrinsic apoptotic pathway. It was shown that CPEs induced apoptosis in U937 cells by increasing caspase-8; however, expression of the death receptors [DR4, DR5, and Fas (a receptor protein of the TFN family)], and proapoptotic ligands such as TFN-related apoptosis-inducing ligand (TRAIL), Fas ligand (FasL), and Fas-associated protein with death domain (FADD) was unchanged (111). Similarly, no reduction in the Fas and FasL proteins was observed in Hep3B cells treated with CPE (119). Further research is required to clarify the precise modulation of extrinsic apoptotic pathways involving cell death receptors by CPEs.

## Inhibition of angiogenesis

It is well established that tumor growth is dependent on angiogenesis—the growth of new blood vessels around cancer tissue needed to supply nutrients and oxygen to tumor cells (147). Because angiogenesis is essential for the growth of different cancers, vascular targeting has been considered as a potential strategy to reduce tumor growth and metastasis.

Flavonoids are antiangiogenic through a variety of mechanisms: they inhibit vascular endothelial growth factor (VEGF) expression, suppress endothelial cell migration, and decrease matrix metalloproteinases MMP-2 and MMP-9 (148).

The antiangiogenic properties of quercetin include inhibition of MMP-2 and MMP-9 secretion from tumor cells as well as inhibition of endothelial cell proliferation and migration (149). Quercetin reduced tube formation of VEGF-stimulated human umbilical vein endothelial cells (HUVECs) by 40% in vitro (150). Luteolin and apigenin are the most potent angiogenesis inhibitors, acting by inhibiting the release of inflammatory cytokine IL-6 and the signal transducer and activator of transcription 3 (STAT3) pathway (149). Hydroxylated PMFs suppress the expression of MMPs and VEGF in colonic tumors. For example, sinensetin inhibited angiogenesis by inducing cell cycle arrest in the G0/G1 phase in HUVEC culture and downregulated the mRNA expressions of angiogenesis genes kinase insert domain receptor (kdrl), transforming protein p21 (hras), and Friend leukemia integration 1 transcription factor (FLI1) in zebrafish (150). Nobiletin inhibited angiogenesis by regulating cell cycle progression through G0/G1 arrest in vivo (150). It also suppressed CD36 expression and decreased the expression of thrombospondin 1—an endogenous inhibitor of angiogenesis—and TGF-β1 (151). Eventually, the expression of VEGF was dramatically modified in DMBA-induced animals by tangeretin treatment (152).

## Inhibition of metastasis

In metastasis, the cancer cells break away from a primary tumor to distal sites in the body. Metastasis involves several distinct steps including secretion of metastasis-inducing proteins, cell detachment at a primary site, migration, adhesion, and invasion at the new site. MMPs such as MMP-2 and MMP-9 are the main proteins that are necessary for metastasis because they break down the extracellular matrix and allow the cancer cells to migrate (153).

The antimetastatic effects of CPE extracted by different methodologies have been tested in a range of cancer cell lines (Table 3). CPEs have been shown to reduce MMP protein expression and activity in A549 (40), DU145 (75), Hep3B (119), and MDA-MB-231 breast cancer cells (154), and in Caco-2, LoVo, and LoVo/ADR colon cancer cell lines (115). In a notable study, quercetin decreased the invasion of murine melanoma cells by suppressing MMP-9 via the PKC activator pathway (155). Genistein prohibited the invasion of triple-negative MDA-MB 231 breast cancer cells in vitro, via downregulation of MMP-9 activity (153, 155). Apigenin, quercine, and luteolin can also inhibit MMP-2 and -9 activities (156). Flavonoids with an increasing number of substitutions or hydroxyl groups showed a stronger inhibitory effect on the activity of MMP-9 and -2 (156, 157). Suppression of the MMP proteins by CPE also was observed in in vivo models for colon (125, 126) and prostate tumors (140).

Like the reduction in MMPs, CPEs reduced concentrations of chemokine receptor CXCR4 together with the human epidermal growth factor receptor 2 (HER2)/neu protein that stimulates CXCR4 expression in MDA-MB-231 cells (154). CPE also suppressed the phospholipase-C gamma-1 (PLCG1) protein required for cell migration in U937 cells (111). Furthermore, vascular cell adhesion molecule-1, which promotes the adherence of cells at new sites, was reduced by *C. unshiu* Marc. peel in MDA-MB-231 cells through inhibition of PKC phosphorylation (112). Many proteins related to metastasis such as reduced epithelial mesenchymal transition (EMT) markers (N-cadherin, vimentin, and fibronectin), EMT-associated transcription factors (Slug and Snail), and activated type I receptors (SMADs) were shown to be downregulated by the Ougan (*C. reticulata* cv. Suavissima) flavedo extract in SKOV3 cells (118).

E-cadherin plays an essential role in cell adhesion, and loss of E-cadherin is associated with a tendency for tumor metastasis (158). An increase in the expression of E-cadherin was observed in colon tumor–bearing mice fed hydroxylated polymethoxyflavones in CPE (125). In another study, the Korean *C. aurantium* L. peel showed antimetastatic properties by preventing the migration and infiltration of A549 cells in an in vitro experiment (40).

## Anti-inflammatory activity

Cancer initiation and proliferation are closely associated with inflammation and, in some cases, infection. Inflammation can facilitate the initiation and progression of normal cells to malignancy through the production of inflammatory oxidants such as inducible nitric oxide synthase (iNOS), myeloperoxidase, eosinophil peroxidase, and NAD(P)H oxidase. Chronic inflammation is associated with carcinogenesis and acts as a driving force for cancer progression (159).

The expression of proinflammatory proteins is reduced by CPE in both in vitro and in vivo models (Table 3). iNOS and inducible-type cyclooxygenase (COX) are enzymes that are induced in response to an oxidative environment. Consequently, overexpression of these enzymes contributes to carcinogenesis through promotion of inflammation (7, 56, 136). CPEs downregulated the expression of iNOS and COX-2 enzymes in human histiocytic lymphoma U937 cell lines, DU145, and murine macrophage RAW264.7 cells (75, 113, 160–162). Reduction in these enzymes by CPEs was also observed in colon, skin, and prostate cancer cell lines in in vivo models (125–127). It is reported that CPEs in RAW264.7 cells reduced nitric oxide that is produced by iNOS (163).

NF-κB activation is an essential factor in inflammation. NF-κB is a heterodimeric protein composed of 5 subunits, and presents in an inactive state in the cytoplasm due to the binding of the inhibitory protein, IκBα (164, 165). Upon chemical signaling for the activation of NF-κB, the IκBα degrades and releases the NF-κB from its inactive state in the cytoplasm. The release of NF-κB allows the translocation of NF-κB subunits p50 and p65 to the nucleus, where they activate the transcription of proinflammatory cytokines, chemokines, adhesion molecules, and enzymes. It is documented that CPE treatment reduced NF-κB activation and the nuclear translocation of its p50 and p65 subunits in RAW264.7, A549, MDA-MB-231, and U937 cancer cells (125, 113, 160,161, 163, 121,166–168).

Likewise, inhibition of NF-κB suppresses a range of downstream genes that include proinflammatory cytokines. Sweet orange peel extract with a high amount of PMFs suppressed the expression of TNF-α, intercellular adhesion molecule 1, IL-1β, IL-6, and IL-8 in inflammation-induced U937 cells (160). The abundances of TNF-α, monocyte chemoattractant protein 1 (MCP-1), IL-6, and phosphorylated p38 proteins were found to be lower in CPE-treated RAW264.7 cells than the control (113).

CPEs also have a suppressive effect on the STAT3 signaling pathway, which is involved in inflammation (75, 169). CPEs reduced the phosphorylation of STAT3 in DU145, PC-3, and prostate cancer cell line M2182 (75). In the same study, Janus-like kinase and a c-Src kinase that mediated the phosphorylation of STAT3 were also found to be suppressed by CPE (125).

The mechanism of action of flavonoids on cancer cells is presented schematically in **Figure 3**. It is highly complex and involves not only certain distinct biological processes but also different modulation of overlapping cell signaling pathways.

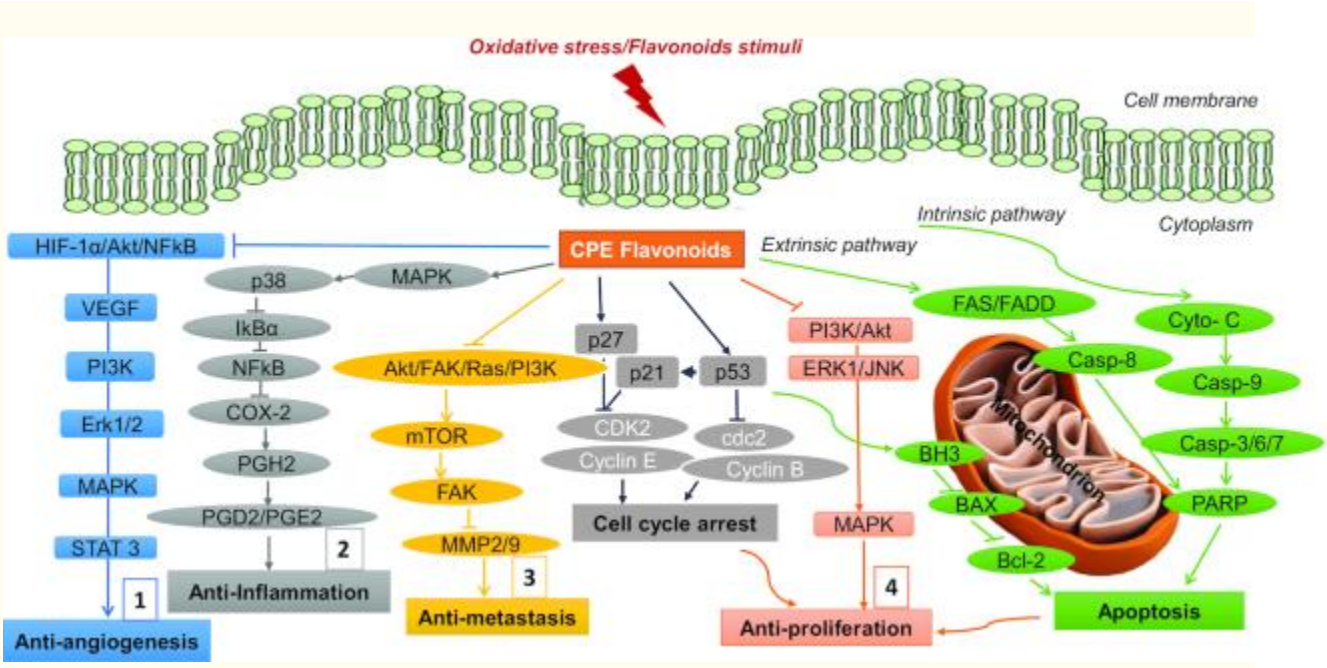


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FIGURE 3

Schematic of the main anticancer molecular mechanism of flavonoids. 1. Antiangiogenesis activity via VEGF by inhibiting HIF-1α/Akt/NF-κB signaling pathways. 2. Anti-inflammation activity by decreasing p38 via MAPK and inhibiting the expression of COX-2. 3. Antimetastasis activity via inhibition of MMP-2/9 by diminishing the Akt/FAK/Ras/PI3K signaling pathways. 4. Antiproliferation activity by inhibiting PI3K/Akt; via cell-cycle arrest in the G0/G1 or G1/S phase by activating p53 and p21, and also inhibiting BAX and Bcl-2; and by increasing cytochrome *c* and activating caspase pathways. Akt, serine specific protein kinase; BAX, Bcl2-associated X protein; Bcl, B-cell lymphoma; BH3, Bcl-2 homology domain 3; Casp, cysteine-aspartic proteases; cdc, cell division cycle; CDK, cyclin-dependent kinase; COX, cyclooxygenase; Cyto-C, cytochrome complex; Erk, extracellular signal-regulated kinase; FADD, Fas-associated protein with death domain; FAK, focal adhesion kinase; FAS, a receptor protein of the TNF receptor family; HIF, hypoxia-inducible factor; IκBα, nuclear factor of kappa light polypeptide gene enhancer; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; mTOR, mechanistic target of rapamycin; PARP, poly ADP-ribose polymerase; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PGH2, prostaglandin H2; PI3K, phosphoinositide 3-kinase; Ras, reticular activating system; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor.

## Functional evidence for citrus anticancer activity in in vivo models

CPE flavonoids have been suggested to play a critical role in cancer prevention and maintaining a healthy lifestyle (207). Individual flavonoids such as apigenin, nobiletin, hesperidin, and tangeretin, all highly enriched in CPE, have demonstrated anticancer activity in preclinical animal models. In addition to single and combined flavonoids, whole CPE has been tested for anticancer activity in rodent models.

A series of studies used preclinical mouse models of colon carcinogenesis to examine the protective effects of crude cold-pressed CPE oil. This oil contained ∼30% PMFs such as nobiletin, sinensetin, tangeretin, and monohydroxylated analogs. When mice were fed a diet containing 0.2% CPE before, during, and after carcinogen treatment (128), the number of aberrant crypt foci (ACF)—histological biomarkers for colon carcinogenesis—was reduced by 34–66% compared with the control. The low incidence of tumor development could be due to the highly potent flavonoids in CPE (102, 128). Feeding mice a diet containing 0.01% or 0.05% hydroxylated PMFs for 4 wk also reduced the total number of large ACF and tumors in colonic tissue by 40–44% compared with controls (125). When mice were fed hydroxylated PMFs for 20 wk, the number of microadenomas was reduced by ≤81% in comparison with controls. Similarly, oral administration of CPE with naringin and hesperidin reduced numbers of ACF by ≤40% compared with the control group in colon tumor–bearing mice (125). Moreover, the addition of CPE (containing methoxylated flavones, including: tetramethoxyflavone, 13.6%; nobiletin, 12.49%; sinensetin, 9.16%; hexamethoxyflavone, 11.06%; heptamethoxyflavone, 15.24%; and tangeretin, 19.0%) at 0.25% or 0.5% to the new Western-style diet reduced the overall colon tumor number by 26–48% and overall tumor volumes by 36–63%, and increased the number of apoptotic cells compared with patients who had the Western-style diet alone (129).

In another study, oral administration of ethanol extract of CPE (*C. junos* Tanaka) at 100 mg/kg/d significantly reduced the size of colorectal adenocarcinoma HT-29 tumor cells through reducing COX-2 expression in xenograft mice (113). Administration of methanol/water extract of dried citrus peel (*C. reticulata* Blanco) at a dose of 1000 ppm in the diet reduced total ACF by 75% compared with the control (121). In a similar study, an in vivo model showed that a 70% aqueous methanol extract of CPE (Korean *C. aurantium* L.) could prevent human lung

(carcinoma) A549 cells migrating to lungs of mice injected with A549 cells via the tail vein (40). These data suggest that CPE had effects on the regulation of apoptosis and cell migration.

In a 2-stage skin carcinogenesis model, mice were treated with 7,12-dimethylbenz(α)anthracene (DMBA) to initiate tumors followed by repeated application of 12-*O*-tetradecanoylphorbol-13-acetate to promote tumor growth. Topical application of CPE, Gold Lotion (the peels of navel oranges, *C. hassaku*, *C. limon*, *C. natsudaidai*, *C. miyauchi*, and satsuma), at 100 μL and 200 μL on the skin reduced the number of papillomas by 25%, tumor incidence by 18%, tumor weight by 65%, and the number of tumors with a diameter >5 mm by 33% compared with controls (127). The epidermal thickening due to the associated inflammation and edema was decreased by 23–33% compared with the control (127).

Apigenin reduced DMBA-induced skin cancers by inhibiting epidermal ornithine decarboxylase, a key enzyme in cancer prevention (208). Nobiletin was effective in preventing skin carcinogenesis by suppression of DMBA and 12-O-tetradecanoylphorbol-13-acetate and decreasing the inflammatory parameters (56). The daily administration of hesperidin for 45 days inhibited DMBA-induced experimental breast cancer formation through modification of phase I and phase II metabolizing enzymes, as well as modulating the xenobiotic-metabolizing enzymes during 1,2-dimethylhydrazine-induced colon carcinogenesis in rats (209). Tangeretin, a PMF, significantly arrested DMBA-induced breast cancer in rats (210). The anticancer activity of CPE (Gold Lotion) was also tested in prostate cancer models. In PC-3 prostate tumor–bearing mice, treatment with CPE by intraperitoneal injection of 1 mg/kg/d reduced the tumor weight by 57% and tumor volume by 79% compared with the control (140, 211). For mice treated with 2 mg/kg/d by oral ingestion, tumor weight was reduced by 86% and tumor size by 94%. The strong anticancer activity was attributed to the high concentration of PMFs and other compounds such as hesperidin. Chu et al. (120) showed that the ethyl acetate extracts from sweet orange peel (50–500 μg/mL) reduced human liver cancer HepG2 growth when tested in an in vivo model and exhibited significant cytotoxicity on HepG2 cells.

Despite the growing number of preclinical animal studies, clinical trials involving CPEs are currently limited to a single study. Naringenin isolated from *C. aurantium* peel (Chinese bitter orange) was tested as a therapeutic on 95 postoperative patients with osteosarcoma (212). The treatment group ($n$ = 47) that received 20 mg/d of naringenin showed significantly reduced osteosarcoma volume compared with placebo controls.

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## Conclusions

Citrus fruits are rich in flavonoid compounds; however, much of the literature to date has focused on the effects of fruit pulp (and juice) consumption rather than examining the rich flavonoid profile of CPE. CPE is an underutilized commercial resource. For instance, the US orange juice industry produces 700,000 tons of peel waste annually (213), which represents nearly 40% of the total weight of the fruit (49). Due to the low cost and current nonuse of the peel by industry, citrus peel represents an untapped nutritional source that is rich in bioactive compounds. There is thus a great deal of potential for the application of citrus fruit peels to create products that counter the effects of oxidative stress and have important health benefits (9).

This review has summarized a selection of the key preclinical and clinical studies that show an anticancer utility for citrus-derived flavonoids. This property is linked to the chemical structures of flavonoids, which can dramatically affect a range of molecular and cellular mechanisms for inhibiting cancer initiation and progression. Overall, citrus flavonoids act not only as free radical scavengers but also as modulators of several key molecular events implicated in cell survival and apoptosis. Flavonoids exhibit a remarkable spectrum of biological activities including anti-inflammatory, anticancer, antiproliferation, antiangiogenesis, antioxidant, cell cycle regulation, and antimetastasis effects.

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## Future Studies

Further studies are needed to address in greater detail the basic science underlying CPE mechanisms, as well as examining pharmacokinetics, pharmacodynamics, and efficacy in a clinical setting. At a fundamental level, there is scope to explore the means by which flavonoids enter cancer cells and potentially accumulate in specific cellular organelles and tissues. This plays into the concept of flavonoid bioavailability, and there has been some discussion regarding innovative methods for enhancing this property (214). Further study could also focus on elucidating signaling pathways by which CPEs can affect critical enzymes such as tyrosine kinases and focal adhesion kinases, PKC, and MMPs.

For clinical translation, trials in both the general population (as health supplements) and in the setting of cancer treatment are needed to build upon cell culture studies and preclinical animal models. Multiple tests indicate that CPEs have a low toxicity profile in vitro and in vivo, making them suitable for further dietary and food product development. Future studies will be required to test the utility of CPEs in a multitargeted pharmacological strategy, either for cancer prevention or as a coadministration in oncological therapies.

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