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Efficacious anti-cancer property of flavonoids from citrus peels

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Abstract

Cancer is one of the two leading human fatal diseases. Drug development for cancer intervention has progressed well in past decades yet existing drugs face many limitations in applications and effectiveness and are often associated with serious of side effects, which can further deteriorate the patients' quality of life. Recent development of natural product based and therapeutically sound anti-cancer agents have gained popularity in the field of functional foods, in which a few have demonstrated efficacy and minimal toxicity toward the prevention and treatment of carcinogenesis. With multiple active molecular components, citrus peels and derived extracts have demonstrated potent efficacious properties against various cancers due in large part to the rich content of flavonoids present in citrus peels. This review summarizes the results of currently available data regarding the *in vivo* anti-cancer activity of citrus peel flavonoids, and identifies opportunities for subsequent human clinical trials to assess preventive and therapeutic effects in the near future.

Keywords: Citrus peels; Flavonoids; Polymethoxyflavones; Efficacy; Cancer

1. Background

Medicinal use of citrus peels, such as aged tangerine peels in south east Asia, can be traced back to 10th century BC. However, the systematic in-depth exploration of the biological activity of citrus peels did not embark until the last decade, when advanced flavonoid profiles in citrus peels were established and isolation of majority of individual flavonoids became available. Since then, a plethora of biological properties important to health and diseases have been identified [1–3]. In addition to cancer prevention and intervention, other biological functions of compounds from citrus peels investigated include inflammation inhibition [4–7], hypolipidemia [8,9], regulation of metabolic syndrome [10–13], delayed onset of Alzheimer's disease [14,15] and more. Characterization of the phytochemical composition of citrus peels with modern analytical technology indicated that citrus peels are an abundant source of polyhydroxyl flavonoids (PHFs) such as hesperidin, neohesperidin and naringin; and almost the sole source of polymethoxyflavones (PMFs) with high content, which are mainly represented by nobiletin, tangeretin, sinesetin, 3,5,6,7,8,3′,4′-heptamethoxyflavone and 3,5,6,7,3′,4′-hexamethoxyflavone [3,16,17].

Research in anti-cancer activity of citrus flavonoids has been majorly focused on *in vitro* experiments to elucidate action mechanisms such as anti-proliferative effects, enzyme inhibition and cancer cell attenuation. PMFs have demonstrated the growth inhibition of human leukemic cell (HL-60) lines [18]. Of PMFs, tangeretin played important inhibitory roles in cancer-cell proliferation and metastasis stage by inhibiting cell adhesion and invasion [19]; showed cell cycle arrest in G1 phase by inhibiting cyclin-dependent kinases (Cdk) and enhancing Cdk inhibitor proteins [20]; inhibited extra cellular-signaling-regulated kinase 1/2 (ERK1/2) phosphorylation and growth of human mammary cancer cells and cytolysis by natural killer cells [21]; repressed induced and constitutively expressed cyclooxygenase-2 (COX-2) in human lung cancer cells [22]. In exploring the anticancer activity of citrus flavonoids, another major PMF, nobiletin was found to have effectively inhibited the proliferation and migration of human umbilical endothelial cells of human prostate,

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skin, breast and colon carcinoma cell lines [23]; reduced AOM-induced cell proliferation in colonic adenocarcinoma cells [24]; suppressed the proliferation, migration and tube formation on matrigel of human umbilical vein endothelial cells stimulated with endothelial cell growth supplement [25]; and attenuated the growth of prostate cancer cells and reduced azoxymethane (AOM)-induced large bowel carcinogenesis in rats [26], to name a few. Multiple biological pathways of anti-cancer mode of action by citrus flavonoids were also studied and summarized [27]. Furthermore, the study of structure activity relationship (SAR) of citrus flavonoids and cancer prevention was linked to the structural similarities between flavonoids and 17β-estradiol, suggesting interaction of flavonoids with estrogen receptors and also with estrogen metabolizing enzymes, such as cytochrome P450 enzymes CYP1A1 and CYP1B1, which are over-expressed in variety of tumor tissues [28].

Conclusions can be made from many *in vitro* studies including those mentioned above that citrus flavonoids exert strong anti-cancer activities. However, the beneficial effectiveness of a drug or a nutrient can only be attested by *in vivo* efficacy studies. In this review, we summarize the *in vivo* anti-cancer effect of bioactive compounds either as single molecules or as a mixture of molecules from citrus peels. The anti-cancer activity of citrus peel flavonoids has been evaluated on several animal models, including cancers of skin [17,29], colon [30–34], prostate [33,35], lung [36], and liver [37] among others.

2. *In vivo* anti-cancer efficacy of phytochemicals in citrus peels

2.1. Skin cancer

In the first reported *in vivo* experiment with citrus polymethoxylated flavonoids, nobiletin effectively inhibited the production of hydrogen peroxide (H_2O_2), attributed to reduced O_2^- generation because of the functional relationship between H_2O_2 formation and O_2^- dismutase or other nonenzymatic reaction [29]. Pretreatment with nobiletin remarkably reduced the weight of edema, thickness of epidermis, number of infiltrated leukocytes and H_2O_2 generation on TPA treated ICR mouse dorsal skin, indicating the efficacy of nobiletin in anti-inflammation and anti-carcinogenesis, because the TPA induced epidermal hyperplasia and edema formation are manifest of inflammatory processes leading to carcinogenesis [29].

Two-stage carcinogenesis skin model was used to characterize the inhibition of tumor promoting effects. The multistage skin carcinogenesis model has initiation, promotion, and progression three distinct stages [38], which serves as a major *in vivo* model for studying the sequential and stepwise evolution of the cancer process by chemical and physical carcinogens. The steps of this standard model include topical application of a single sub-carcinogenic dose of skin carcinogen, such as DMBA that causes irreversible DNA damage; then repeated application of promoters such as most commonly used TPA to induce cell proliferation and inflammation. This promotes the selective clonal expansion of initiated epidermal cells and leads to the formation of multiple squamous papillomas. Also in the same *in vivo* experiment, nobiletin at dosages of 0.16 and 0.32 μmol/L effectively inhibited the growth of mouse skin tumor initiated by DMBA and promoted by TPA in a dose-dependent manner [29]. The tumor incidence at 20 weeks was reduced by 33.5–43.3% in nobiletin treated groups *vs* in the control group. The average number of tumors per mouse was inhibited by 61.2% (0.80 ± 0.48) at dose of 0.16 μmol/L and 75.7% (0.50 ± 0.22) at dose of 0.32 μmol/L, respectively [29].

In the same skin cancer model, the topical application of a mixed citrus peel extract (CPE) also effectively inhibited molecular biomarkers of skin inflammation and attenuated TPA-induced skin tumor formation by reducing both the tumor incidence and tumor multiplicity of papillomas at 20 weeks, which demonstrated the efficacious anti-tumor effect of molecules from the citrus peel extract (CPE) and the capability of preventing inflammation-associated skin tumorigenesis [17]. The untreated positive control group of mice had 16 ± 3 papillomas per mouse and a 100% incidence of skin tumors at 20 weeks, whereas no tumors were observed in the negative control group following acetone application. However, when 100 μL of the CPE was applied to the shaven backs of mice 30 min prior to each TPA application, the average number of papillomas per mouse was 12 ± 4 (25% reduction compared to positive group). In addition, the tumor incidence was found to be 100% in positive group whereas extract-treated group showed significantly decreased at 81%. The number of papillomas (≥5 mm in diameter) per mouse was significantly decreased in the CPE treated group. In addition, in the positive control group in which mouse skin tumors were initiated by DMBA and promoted by TPA for 20 weeks, the protein expression of ornithine decarboxylase (ODC), COX-2, and VEGF (vascular endothelial growth factor) was apparently increased compared to healthy skin tissue. However, treatment with the CPE resulted in a strong reduction of ODC, COX-2, and VEGF protein levels in skin tumors. ODC is involved in cancer proliferation and both COX-2 and VEGF contribute to angiogenesis, hence we suggest that citrus flavonoids reduced tumor size by inhibiting the tumor growth and angiogenesis [17].

2.2. Colon cancer

Colorectal cancer (CRC) has high rates of mortality and morbidity and also has increasing prevalence even with current choices of diagnosis and medication [30]. In experimental models, development of CRC from normal colonic epithelium includes several distinct steps, *i.e.* colonic crypt hyperplasia, dysplasia, adenoma, adenocarcinoma and distant metastasis [30,31]. The formation of aberrant crypt foci (ACF) in early stage of the progression is widely considered a histological biomarker of colon tumorgenesis [31]. ACF also occurs very frequently in colon cancer patients, which proposed as a putative pre-neoplastic lesion [31]. Furthermore, increased number of incidence and multiplicity of ACFs are closely associated with CRC development [30,31]. The tumorigenesis of colon cancer is involved in various genetic and molecular changes in cell proliferation, inflammation, resistance to apoptosis and tumor angiogenesis.

The *in vivo* study of suppressing colon cancer by citrus peel flavonoids has drew much attention in past decade. In a model of mouse carcinogenesis, five week old mice ICR mice had a single AOM intraperitoneal injection followed by drinking water containing 1% DSS (dextran sulfate sodium) for seven days. A major citrus flavonoid, nobiletin (100 ppm) was fed in a diet for 17 weeks [32]. At the end of 20 weeks, the incidence and number of colon tumors and serum concentration of adipocytokines were measured. Data has shown that the nobiletin feeding group dramatically reduced both the incidence and the number of carcinomas in colon tissue. The concentration of serum leptin in AOM/DSS treated mice was six times higher than that in control group of mice, whereas it was found no significant differences in the concentrations of triglycerides, adiponectin and IL-6. However, the serum leptin level was reduced by 75% in the group of nobiletin ingestion mice, suggesting that the chemopreventive effect of nobiletin against colon tumorigenesis be through the reduction of high level of serum leptin that was found to be a promoter of colon carcinogenesis in mice [32].

To examine the influential factor of obesity on colorectal cancer, the animal model of db/db mice, genetically altered model with the genotype of obesity and diabetes mellitus was used [33]. The mice of strain have developed hyperleptinemia, hyperinsulinemia, hyperglycemia, hyperlipidemia and hypercholesterolemia. Obesity along with elevated levels of serum cholesterol, triacylglycerols, glucose, insulin and leptin is reported to be highly susceptible to colon carcinogenesis due in part to the proliferation of cancer cells. Leptin was suggested previously to act as a mitogenic factor in colon carcinogenesis of mice [32]. Hence db/db mouse model plays a useful role in elucidating the relationship between colon tumorigenesis and obesity/diabetes [33]. Nobiletin treatment of AOM-induced preneoplastic lesion in db/db mice significantly reduced the colon ACF incidence by 68%, particularly noteworthy the number of large ACFs that are closely associated with colonic adenocarcinoma. Nobiletin feeding did not lower the level of leptin in the db/db mice treated with AOM, but the serum levels of IGF-1 were dramatically decreased. It was postulated that such reduction may lead to the suppression of proliferation in preneoplastic lesions. Results from this *in vivo* study provided evidence that citrus peel flavonoid, nobiletin is a potent inhibitor of early stage colon carcinogenesis in obese mice *via* the reduction of proliferation [33].

In addition to nobiletin, a mixture of natural citrus flavonoids was recently examined for its *in vivo* anti-colonic cancer activity [34]. In the model of AOM-induced colonic tumorigenesis, oral feeding of mixed citrus peel extract (CPE) decreased the total number of aberrant crypt foci (ACF), particularly large ACFs in colonic tissues of mice. Both gene and protein expression of iNOS and COX-2 were suppressed by the CPE treatment. The *in vivo* data have revealed for the first time that CPE, the mixed extract of citrus peels is an effective anti-tumor agent mechanistically down-regulating the protein levels of iNOS, COX-2, ODC, VEGF, and MMP-9 (matrix metallopeptidase-9) in colonic tissues of mice, suggesting a mechanism underlying its therapeutic function [34]. In the AOM model, formation of ACFs in early stage is considered to be a histological biomarker

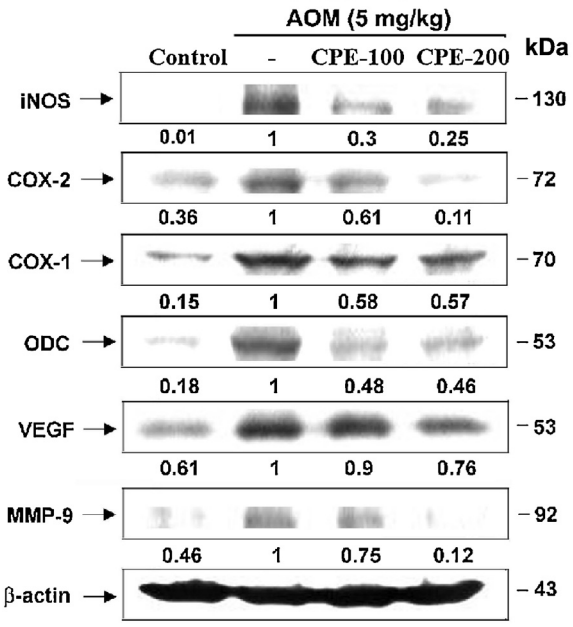

Fig. 1. Inhibitory effects of oral administration of CPE on AOM-induced protein levels in colorectal tissue [34]. Protein lysates from normal colonic mucosa and ACF of each group were extracted and subjected to Western blot analysis for iNOS, COX-2, COX-1, ODC, VEGF and MMP-9 protein levels.

of colonic tumor development. Large ACFs are dysplastic, with histological features of micro adenomas that are associated with an increased risk for malignant progression [35]. In the efficacy study of the citrus peel extract against CRC, the number of ACFs with CPE treated mice is remarkably lower than AOM-treated group of mice. Surprisingly, the number of larger ACFs was targeted first and reduced by about 40% compared to that in the AOM-treated positive control group ($P < 0.05$).

The development of CRC involves various genetic and molecular changes in cell proliferation, inflammation, resistance to apoptosis, angiogenesis, and metastasis. For instance, over expression of iNOS and COX-2 enzymes contributes to the promotion of tumorigenesis by induction of inflammation, abnormal cell proliferation, and decreasing apoptosis. Hence, the assessment of the CPE on AOM-induced inflammatory (iNOS and COX-2), proliferative (ODC) and angiogenic (VEGF and MMP-9) molecule expression in AOM-treated mouse colon found dramatically increased expression of inflammatory enzymes (iNOS and COX-2) in AOM alone treated mice. As shown in Fig. 1, CPE oral administration resulted in a remarkable reduction of iNOS and COX-2 protein level in mouse colon and the gene expression of both i*NOS* and *COX-2* genes in a dose-dependent manner compared with the AOM-treated positive control group [34]. These results suggest that the mechanism of *in vivo* chemopreventive efficacy of CPE includes anti-inflammation, anti-proliferation, and anti-angiogenesis in AOM-induced colonic tumorigenesis.

2.3. Prostate cancer

The *in vivo* anti-cancer property of citrus flavonoid nobiletin was also demonstrated in chemopreventive effects

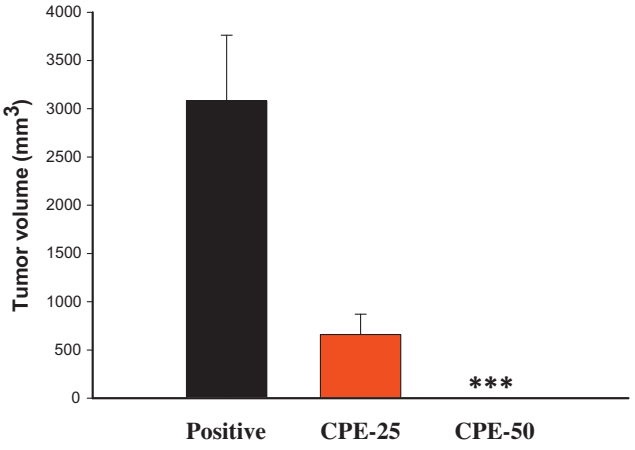

Fig. 2. Growth of PC-3 tumor xenografts in nude mice was reduced by *i.p.* treatment with 25 and 50 μL of mixed CPE, respectively.

of colon and prostate cancer induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), which was administered intragastrically to six week old F344 male rats twice a week for 10 weeks [36]. In the medication group, diet containing 0.05% nobiletin was given to the rats for 50 weeks. Data has shown that nobiletin significantly reduced the weights of prostates and testes, reduced the incidence and multiplicity of adenocarcinomas by 50% and 64%, respectively, and furthermore, significantly reduced the total number of colonic ACF [36], which demonstrated the *in vivo* effectiveness of citrus flavonoid nobiletin in chemoprevention of both prostate and colon carcinogenesis [36].

Prostate cancer is one of the most prevalent male diseases after cardiovascular mellitus [37]. Mortality from prostate cancer has decreased recently owing to improved early diagnosis and selection of more efficacious medicinal substances [38], but it remains one of the most common malignancies with a high incidence, an increase in hormone-resistant types, and metastasis [39]. The human prostate tumor xenograft model has been widely used in cancer therapeutic research and drug development [40].

The mixed citrus peel extracts, CPE was also tested in immune-deficient mice bearing human prostate cancer cell line PC-3 tumor xenografts [41]. After the establishment of palpable tumors, animals received intraperitoneal (*i.p.*) injections of two dosages of CPE (25 μL and 50 μL) five times per week for 23 days. Apparent inhibition of PC-3 xenograft tumor growth was observed even at lower dose (25 μL). The prostate xenograft tumor was nearly eliminated in the group treated with the higher dose of 50 μL (Fig. 2). Consistent with this observation, both tumor weight and tumor size were remarkably decreased in both medication groups. Mice in the control group showed an average tumor weight of 1.66 g and it was reduced by 57% in the group treated with the lower dose. Similarly, the average tumor volume was five times lower in the low dose group than in the control group. Hence, tumor weight and tumor volume were dramatically reduced by *i.p.* injection of CPE in PC-3 xenograft model [41]. Oral dosing was also investigated in the evaluation of therapeutic effects of this formulated CPE. Animals were orally administered with 50 μL or 100 μL of CPE in two groups five times per week for 21 days. The body weight of mice in each group at the end of the study was not much different: (20.41 ± 2.11) g in positive control group *vs* (21.22 ± 0.53) g in 50 μL of CPE-treated group *vs* (21.34 ± 0.90) g in 100 μL of CPE-treated group. After 21 days of CPE oral ingestion, the growth of PC-3 xenograft tumors was dramatically inhibited in both incidence and volume. The average tumor size in positive group was (1021.9 ± 476.2) mm^3, whereas in the CPE-treated groups, tumor size was dramatically reduced to (57.7 ± 42.0) mm^3 (50 μL CPE) and (69.1 ± 9.9) mm^3 (100 μL CPE), respectively. There was no dose-response between 50 and100 μL groups being observed because the CPE was highly effective even at the low dose (50 μL) consumption. These results provided evidence that oral administration of CPE also exerted effective efficacious activity against prostate carcinogenesis. Therefore, in evaluating the efficacy of the mixed citrus peel extract (CPE) against prostate cancer in a xenograft mouse model, we found that treatment with CPE by both *i.p.* injection and oral administration dramatically reduced the tumor weight and size without any observed toxicity. This inhibitory effect was accompanied by a down-regulation of proteins associated with inflammation (iNOS and COX-2), metastasis (MMP-2 and MMP-9), angiogenesis, and induction of apoptosis in prostate tumors [41].

2.4. Lung cancer

Lung cancer remains the leading cause of cancer deaths globally. The current treatment of lung cancer is not effective as expected. It claims more lives than do colon, prostate, breast and ovarian combined for past ten years [42]. One of the major citrus flavonoids, nobiletin has demonstrated its anti-lung cancer activity in a murine tumor model [43]. In this model, six-week-old male nude mice were injected with A549 cells. Treatment began when the tumor size reached about 75 mm^3 after development for 20 days. 3 dosages were applied in the nobiletin groups, low dose of 100 mg/kg, medium dose of 200 mg/kg and high dose of 300 mg/kg. Cyclophosphamide (60 mg/kg) was used as control group and non-medicated inoculated mice as positive groups. The results showed the inhibition percentage of tumor growth as 51.3%, 41.3%, 29.6% and 57.6%, respectfully corresponding to high (300 mg/kg), medium (200 mg/kg), low (100 mg/kg) doses of nobiletin and cyclophosphamide (60 mg/kg). Furthermore, there has been found no sign of toxicity judging from body weight of animals in nobiletin treated groups. The *in vitro* anti-cancer mechanisms of nobiletin found prior to the *in vivo* study included apoptosis – nobiletin induced cell cycle arrest at G2/M phase in A549 cells, inhibition of Bcl-2 protein expression, increase of Bax and p53 protein expression and elevated protein ratio of Bax/Bcl-2 [43].

2.5. Liver cancer

Liver cancer is one of the leading causes of death, particularly in South East Asia. Citrus flavonoid, nobiletin has demonstrated anti-liver cancer activity both *in vitro* and *in vivo* [44,45]. In the

flow cytometric analysis, nobiletin blocked the cell cycle arrest at G2/M phase. The *in vivo* antitumor activity of nobiletin was performed on KM mice inoculated with H22 cells. The mice were gavage fed daily for 11 days with nobiletin at low of 125 mg/kg, medium of 250 mg/kg and high of 500 mg/kg. Nobiletin significantly inhibited tumor growth at high dose group and it was also found to have down-regulated the *Bcl*-2 and *COX*-2 expression and up-regulated the expression of *Bax* and caspase-3 in HCC cell line (SMMC-7721 cells) in Western blot [45]. The ratio of Bcl-2/Bax was significantly reduced at dosages of 250 and 500 mg/kg. Nobiletin also effectively suppressed the expression of *COX*-2 [45].

3. Citrus flavonoids and their biological activity

Citrus and citrus peels contain common flavonoids, such as hesperidin, naringin, neohesperidin, narirutin, eriocitrin, didymin and rutin among others [27,46,47]. A number of studies have demonstrated the biological properties of these citrus flavonoids including anti-carcinogenic, anti-oxidant and anti-inflammatory properties that promote and benefit human health [46–49]. In addition to citrus flavonoids, citrus peels are also the sole and rich source of polymethoxylated flavonoids, which were found to exert many biological properties, particularly anti-cancer and anti-inflammatory activity [1,4,5,7]. Recent studies have also demonstrated potent anti-carcinogenic and anti-inflammatory efficacy of 5-demethylated polymethoxyflavones in single molecules [5,50,51] or in multiple 5-demethylated polymethoxyflavones [52]. The natural content of 5-demethylated polymethoxyflavones in citrus peels is low in percentage, but it has been confirmed that they have more potent biological activity than their non-demethylated counterparts, such as anticancer activity [50–52].

In essence, there are three subclasses of citrus flavonoids exiting abundantly in citrus peels, namely, polyhydroxyflavonoids, polymethoxyflavonoids and mixed substituted flavonoids with both hydroxyl and methoxyl groups, particularly 5-demethylated polymethoxyflavnoids. These flavonoids have demonstrated effective anti-cancer property both *in vitro* and *in vivo*, either in a form of individual compounds or in a mixture of citrus flavonoids. The anti-cancer study of these flavonoids has progressed well in recent years owing albeit in the initial steps to the modern chemical analysis and isolation and the biological activity testing. However, with the exception of nobiletin, the relationships between each individual flavonoid in citrus peels and its bioactivity such as anti-carcinogenesis remain untouched to some extent. Relationships among the naturally proportioned flavonoids in citrus peels and their biological activities are even more complex and unexplored.

4. Conclusion

In summary, the *in vivo* studies provide compelling evidence that flavonoids from citrus peels, such as nobiletin and citrus peel extract (CPE) demonstrated potent anti-tumor activities in cancers of skin, colon, prostate, lung and liver. The therapeutic mechanism may include inhibition of inflammation, proliferation, angiogenesis, and induction of apoptosis. A characteristic and unusual feature of flavonoids from citrus peels is their high concentration of a diverse assortment of polyhydroxylated flavonoids, polymethoxyflavones, and other flavonoids. Nobiletin, a representative of citrus polymethoxyflavones, expressed potent efficacious activity against carcinogenesis. In view of other studies examining the impact of individual flavonoids such as polyhydroxylated flavonoids (PHFs) or polymethoxyflavones (PMFs) and related flavonoids [1], it is possible that the biological properties of mixed flavonoids from citrus peels summarized above are due to an additive or synergistic interaction of the complex mixture of phytochemicals, which may provide enhanced anti-cancer efficacy. The detailed mechanisms by which these flavonoids in citrus peel exert optimal benefits remain to be further elucidated. However, the evidence presented in this review illustrates that citrus flavonoids could prove to be an effective anti-cancer agent, especially against skin, colon, prostate, lung and liver cancer and encourage future research to evaluate its efficacy in human clinical trials.

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