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# Chemistry and health effects of furanocoumarins in grapefruit

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## ABSTRACT

Furanocoumarins are a specific group of secondary metabolites that commonly present in higher plants, such as citrus plants. The major furanocoumarins found in grapefruits (*Citrus paradisi*) include bergamottin, epoxybergamottin, and 6′,7′-dihydroxybergamottin. During biosynthesis of these furanocoumarins, coumarins undergo biochemical modifications corresponding to a prenylation reaction catalyzed by the cytochrome P450 enzymes with the subsequent formation of furan rings. Because of undesirable interactions with several medications, many studies have developed methods for grapefruit furanocoumarin quantification that include high-performance liquid chromatography coupled with UV detector or mass spectrometry. The distribution of furanocoumarins in grapefruits is affected by several environmental conditions, such as processing techniques, storage temperature, and packing materials. In the past few years, grapefruit furanocoumarins have been demonstrated to exhibit several biological activities including antioxidative, -inflammatory, and -cancer activities as well as bone health promotion both *in vitro* and *in vivo*. Notably, furanocoumarins potently exerted antiproliferative activities against cancer cell growth through modulation of several molecular pathways, such as regulation of the signal transducer and activator of transcription 3, nuclear factor-κB, phosphatidylinositol-3-kinase/AKT, and mitogen-activated protein kinase expression. Therefore, based on this review, we suggest furanocoumarins may serve as bioactive components that contribute, at least in part, to the health benefits of grapefruit.

## 1. Introduction

Citrus fruits, belonging to the family Rutaceae, genus *Citrus*, are believed to originate from certain regions of Southeast Asia and are mainly cultivated in the regions in the Northern Hemisphere [1]. Citrus crops are one of the major fruit crops in the world and it is estimated that more than 135 million tons were produced in 2013 [2]. The leading citrus fruit-producing countries in the world include China, Brazil, the United

States, India, Mexico, and Spain, which collectively account for two-thirds of the global production [3]. Citrus taxonomy remains controversial. However, the four core ancestral *Citrus* taxa including *Citrus medica* (citron), *Citrus reticulata* (mandarin), *Citrus maxima* (pummelo), and *Citrus micrantha* (papeda) have been recognized as the ancestors of all cultivated citrus fruits [4,5]. The major *Citrus* fruits consumed worldwide are oranges, mandarins, lemons, grapefruits, as well as limes. Owing to a pleasing flavor and aroma as well as desirable taste, citrus species are widely consumed as fresh or used as raw materials for juice. Citrus fruits are not only a particular rich source of vitamin C, but are also abundant in nutrients such as dietary fiber, sugar, and minerals. Along with a high nutrition value, the secondary metabolites of citrus fruits, including flavonoids, limonoids, and coumarins, they are also known to possess several health benefits such as antioxidative, anti-inflammatory, anticancer and neuroprotective activities.

Grapefruits (*Citrus paradisi*) are medium-sized, subtropical fruit trees that belong to the family of Rutaceae. Grapefruit, a hybrid of pomelos (*C. maxima*) and sweet oranges (*Citrus sinensis*) was first discovered in the 18th century. Different varieties of grapefruits vary in hue from white to red depending on the presence or absence of lycopene [6]. According to the data from the Food and Agricultural Organization of the United Nations, China and the United States are the leading grapefruit producers worldwide. In China, a total of 3.8 million metric tons of grapefruit was reportedly produced in 2013 [3]. The major varieties of grapefruit include Pink, Ruby Red, Star Ruby, Thompson, and White Marsh. Owing to several bioactive phytochemicals, such as flavonoids, carotenoids, coumarins, and organic acids, grapefruit possesses several health-promoting properties such as anti-inflammatory, -cancer, and -obesity effects [7–9]. Flavonoids are considered the most important bioactive components present in grapefruit. The major flavonoids found in grapefruit, including hesperetin, naringenin, narirutin, and didymin, have been extensively studied both *in vitro* and *in vivo* to confirm their role in benefiting human health [10,11]. Although these phytochemicals exhibit bioactive activities, some compounds have been shown to interact with numerous medications causing adverse effects known as the "grapefruit juice effect." Furanocoumarins and flavanones are the major culprits responsible for grapefruit juice causing these drug interactions. Several studies have reported that furanocoumarins present in grapefruit interact with medications by interfering with the haptic and intestinal enzyme cytochrome P450 [12]. Several studies have developed different processing methods for the removal of furanocoumarins from grapefruits such as heat treatment, UV irradiation, and autoclaved fungi [13–15]. Until now, numerous comprehensive reviews summarized the grapefruit juice–drug interaction mechanisms mainly focusing on discussion of the adverse effects of furanocoumarins [16–18]. Although furanocoumarins can cause undesirable effects because of interactions with certain medications, recent evidence has emerged from several *in vitro* and *in vivo* studies suggesting that furanocoumarins possess additional biological activities, such as antioxidative, -proliferative, -inflammatory, and bone health promoting effects. As

grapefruit juice is one of the most popular fruit juices worldwide, grapefruit furanocoumarins deserve more attention with regard to their health benefits. Thus, this review first summarizes the biosynthetic pathway, analytical methods, and distribution of grapefruit furanocoumarins, and then provides a comprehensive view of their health benefits.

## 2. Biosynthetic pathway of furanocoumarins in grapefruit

Furanocoumarins, a subclass of organic chemical compounds, are the secondary metabolites produced in citrus and are involved in the plant's defenses against insects, pathogens, and other organisms [19]. Their structure is characterized by a furan ring attached to carbon 6 and 7 (linear type) or 7 and 8 (angular type) of a benzo-α-pyrone (coumarin). Linear furanocoumarins are practically distributed into four families of higher plants that include Rutaceae, Moraceae, Leguminosae, and Apiaceae, whereas the angular furanocoumarins are primarily confined to Apiaceae and Leguminosae [19]. The most abundant linear furanocoumarins existing in higher plants are psoralen, xanthotoxin, bergapten, and isopimpinellin.

Citrus plants synthesize both coumarins and furanocoumarins with grapefruit considered the major dietary source of furanocoumarins in the Western diet. It is estimated that the average consumption of furanocoumarins in the United States and Germany is 1.3 mg/d and 0.562 mg/d, respectively, and grapefruit juice contributes to ~73% of furanocoumarin intake from foods [20,21]. The major furanocoumarins found in grapefruits are bergamottin, epoxybergamottin, and 6′,7′-dihydroxybergamottin. The pathway of furanocoumarin biosynthesis was confirmed and characterized using radiolabeled compounds during 1960–1980 [22–24]. This biosynthetic pathway in higher plants use umbelliferone, also known as 7-hydroxycoumarin, as the precursor of these furanocoumarins. In the first step of coumarin synthesis, several phenylpropanoid intermediates are synthesized from phenylalanine (Figure 1). Through a deamination reaction by phenylalanine ammonia-lyase, phenylalanine is first converted to *trans*-cinnamic acid. This is subsequently catalyzed by cinnamate 4-hydrolase (C4H), an essential enzyme involved in phenylalanine metabolism, to form *p*-coumaric acid. C4H, a member of cytochrome P450 monooxygenase from CYP73A family, can catalyze monooxylation of various substrates within organisms [25,26]. *p*-Coumaric acid is further catalyzed through thioesterification by 4-coumarate-CoA ligase, and *p*-coumaroyl-CoA is then produced. In the phenylpropanoid pathway, coumaroyl-CoA is the pivotal intermediate of not only coumarins, but also a broad range of metabolites, such as flavonoids, anthocyanins, tannins, and lignans [27,28]. Next, the *ortho*-hydroxylation of *p*-coumaroyl-CoA catalyzed by cinnamoyl-CoA 2′-hydroxylase (C2′H) leads to synthesis of 2,4-dihyroxycinnamoyl CoA. *ortho*-Hydroxylation is a key step of coumarin biosynthesis, and a previous study of *Lavandula officinalis* demonstrated that *para*-hydroxylation was a prerequisite for *ortho*-hydroxylation [23]. Finally, nonenzymatic lactonization results in
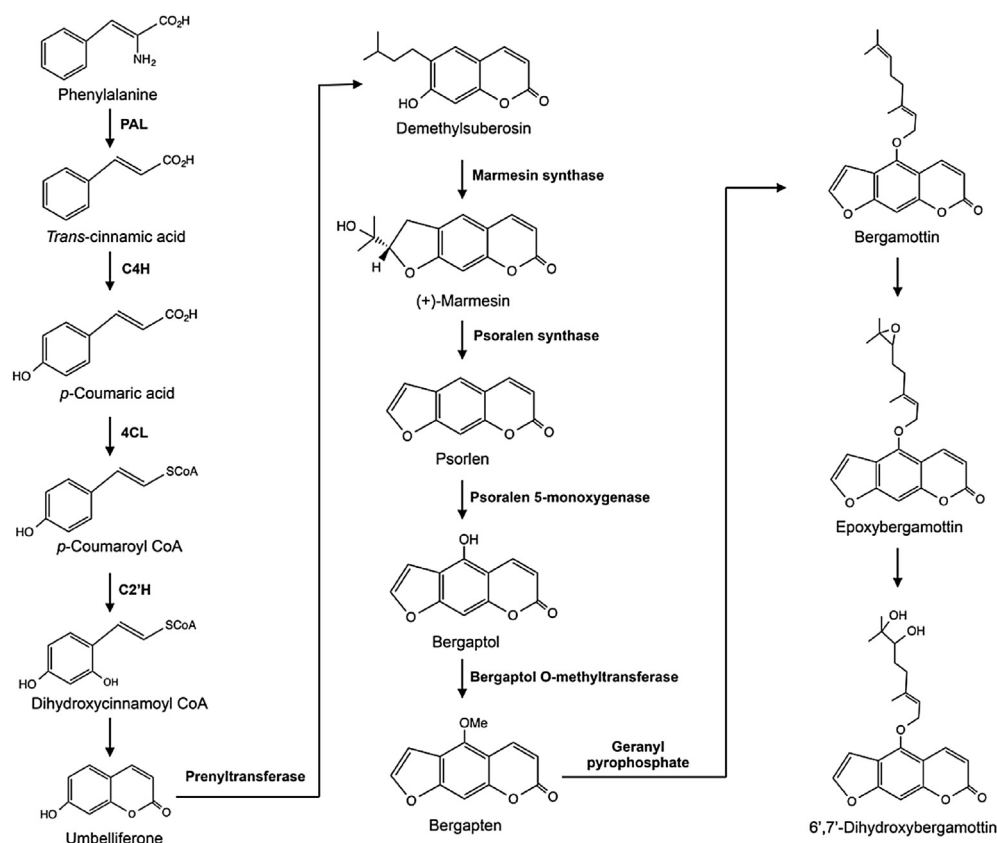
**Figure 1 − Biosynthetic pathway of 6′,7′-dihydroxybergamottin in citrus fruits. 4CL = 4-coumarate-CoA ligase; C2′ H = cinnamoyl-CoA 2′hydroxylase; C4H = cinnamate 4-hydrolase; PAL, phenylalanine ammonia-lyase.**

umbelliferone formation [19]. These enzymatic reactions involved in the formation of umbelliferone from phenylalanine are illustrated in Figure 1.

Umbelliferone, which belongs to the coumarin family, is an ubiquitous compound found in higher plants. It is also the parent compound responsible for not only furanocoumarin formation, but also methoxylated coumarins such as scopoletin and its glycoside derivative [29]. The prenylation catalyzed by the 6′-prenyltransferase occurring in C6 position of umbelliferone yields linear furanocoumarins (demethylsuberosin), whereas the 8′-prenyltransferase produces angular furanocoumarins (osthenol). The first plant prenyltransferase characterized *in vitro* by Dhillon and Brown [30] was isolated from *Ruta graveolens*, which produced psoralen only. This enzyme is identified as umbelliferone:dimethylallyl pyrophosphate dimethylallyl transferase existing in chloroplasts [31]. In the linear furanocoumarin biosynthetic pathway, demethylsuberosin is transformed to (+)-marmesin and further converted to psoralen by two consecutive cytochrome P450-dependent monooxygenases, the marmesin synthase and the psoralen synthase. The mechanism of the conversion of demethylsuberosin to marmesin was first proposed in 1970 [32]. Enzymatic oxidation of dimethylsuberosin catalyzed by marmesin synthase produces the corresponding epoxide, 6-(2-methyl-2,3-epoxybutyl)-umbelliferone, and subsequently hydration of the epoxide produces the corresponding diol and further cyclization formation of (+)-marmesin. After eliminating acetone and one hydrogen catalyzed by psoralen

synthase, (+)-marmesin is converted to psoralen. It is noteworthy to mention that psoralen is specific for (+)-marmesin, and therefore, the (−)-steroisomer, nodakenetin, cannot be its substrate.

Psoralen serves as the parent compound of linear furanocoumarins frequently transformed into hydroxylated forms by cytochrome P450 monooxygenases. The hydroxylation of psoralen most likely occurs at either 5- and/or 8-position of bergaptol (5-hydroxypsoralen), xanthotoxol (8-hydroxypsoralen), and 5,8-dihydroxypsoralen formation [2]. The formation of bergaptol, catalyzed by psoralen 5-monooxygenase in the presence of oxygen and nicotinamide adenine dinucleotide phosphate, has been characterized as inducible cytochrome P450 monooxygenase from microsomes of cultured *Ammi majus* cells [33]. However, only small quantities of bergaptol can be found in in grapefruits because this hydroxylated furanocoumarin is subsequently transformed into bergapten by bergaptol *O*-methyltransferase. Previous studies have successfully isolated bergaptol *O*-methyltransferase from both *Ruta graveolens* and cultured parsley cells by chromatographic methods and further confirmed that this enzyme solely catalyzes the methylation of the 5-hydroxy group of bergaptol, whereas xanthotoxol *O*-methyltransferase specifically catalyzes the 8-hydroxy group of xanthotoxol [34,35]. The sequence of bergaptol 5-methyltransferase cDNA cloned from elicitor-treated cells of *A. majus* shared 79% similarity with the sequence of heterologous caffeic acid 3-*O*-methyltransferase at the polypeptide level. Their study also

confirmed that bergaptol 5-methyltransferase was highly specific for bergaptol, and the C8 corresponding isomer xanthotoxol cannot function as its substrate [36]. In the final step, geranyl pyrophosphate (GPP) attaches to the bergapten to form bergamottin. Prenylation is a key step to form coumarin derivatives through adding a prenyl chain. In addition, begaptol also can transform into bergamottin through the prenylation reaction. Recently, Munakata et al [37] characterized the coumarin-specific prenyltranferases from the microsomes of the flavedo of lemon peel where large amounts of prenylated coumarins accumulate. Among several coumarin substrates, bergaptol 5-O-geranyltransferase had the strongest activities catalyzing bergaptol to form bergamottin when GPP was used as a prenyl donor.

## 3.    Furanocoumarin analytical method

Coumarins and furanocoumarins are widely distributed in plants, especially in the families Apiaceae, Rutaceae, Moraceae, and Leguminosae. At least 1300 coumarins have already been identified, and several new coumarins are being identified each year [38]. The predominant furanocoumarins present in grapefruits include bergaptol, bergapten, bergamottin, expoxybergamottin, and 6′,7′-dihydroxybergamottin. Furanocoumarins exhibit several physiological effects, the most notable being interactions with certain medications. Furanocoumarins' interactions with some drugs result in undesirable effects by interfering with hepatic and intestinal enzyme cytochrome P450. Consequently, several studies have also reported beneficial furanocoumarin bioactivities such as antioxidative and -tumor effects. These physiological effects caused by furanocoumarins suggest that quantitation of their concentrations in grapefruit is important. Thus, many analytical methods have been developed for identification and quantitation of grapefruit furanocoumarins (Table 1).

Prior to analyzing furanocoumarins, the crucial first step is extraction of the target compounds from plants for further purification and identification. Selection of the extraction method depends on the property of the target compounds of interest and plant matrices. Additionally, many factors substantially affect the extraction yield, such as solvent system and extraction techniques. Coumarins, the precursors of furanocoumarins, can be isolated from plants by extraction with different solvents such as ethanol, methanol, benzene, and chloroform. Additionally, extraction with petroleum ether provides excellent yield for furanocoumarins [47]. Among the numerous extraction techniques, exhaustive extraction preformed on a Soxhlet apparatus is the most accurate method to extract furanocoumarins from plants [48]. The extraction yield of furanocoumarins in *Archangelica offinalis* using different extraction methods has been compared by Waksmundzka-Hajnos et al [49]. Their results revealed that accelerated solvent extraction using methanol or petroleum ether as the extraction solvent resulted in the highest yield of furanocoumarins, and the yield from other extraction methods such as ultrasonication and microwave-assisted solvent extraction were comparable with that from the exhaustive extraction method. It is noteworthy that microwave-assisted solvent extraction in a closed system may cause transformation of furanocoumarins because of the microwaves.

Liquid–liquid extraction is the most common extraction method for furanocoumarins in grapefruit juice. It is necessary to form two separate layers; therefore, this extraction method is limited by the extracting solvent as it must be immiscible with the aqueous juice. Thus, highly polar solvents such as methanol, ethanol, and acetone are not applicable for liquid–liquid extraction. Although chloroform provides a good yield of extracted coumarins and furanocoumarins, many studies still used ethyl acetate as extraction solvent because of its low toxicity. For example, VanderMolen et al [50] used ethyl acetate to extract furanocoumarins and flavonoids from grapefruit juice and developed a rapid quantitative method with good precision, accuracy, and sensitivity. As for grapefruit peel and pulp, solid–liquid extraction is the typical method used to extract furanocoumarins from these citrus fruits, with methanol most often used as the extracting solvent. Several studies have used methanol to extract furanocoumarins from citrus fruits prior to measuring their concentrations by high-performance liquid chromatography (HPLC). Peroutka et al [51] have compared the extraction efficiency of different solvents including acetonitrile, methanol, and ethyl acetate on extracting bergamottin from the peel and pulp of citrus fruits. Their result indicates that methanol is the optimum solvent for extraction of coumarins and furanocoumarins from citrus fruits. Similarly, coumarins and furanocoumarins can be extracted by methanol/water (80:20) from the peel and pulp of 61 *Citrus* species with results showing the major furanocoumarins present in grapefruit peel and pulp to be 6′,7′-dihydroxybergamottin, epoxybergamottin, and bergamottin [2].

After solvent extraction, solid-phase extraction (SPE) is often used for purification of the extracted mixture to purify the target compounds of interest prior to HPLC analysis. The extraction recoveries of standard aqueous bergamottin and bergapten, the two major furanocoumarins present in grapefruits, have been compared by different SPE sorbents [41]. The highest extraction recovery was obtained when a reverse-phase DSC-18LT cartridge was used as the SPE sorbent. Because of the semipolarity of furanocoumarins, normal-phase sorbents can also be used to isolate furanocoumarins from grapefruit juice. A silica gel column has been used to isolate 6′,7′-dihydroxylbergamottin, bergamottin, and paradisin A individually from the ethyl acetate extraction of grapefruit juice prior to nuclear magnetic resonance (NMR) spectroscopy analysis [52]. Along with SPE, flash column chromatography also provides reasonable and rapid separation of furanocoumarins in grapefruits. Chebrolu et al [53] developed flush column chromatography methods using either C18 or silica gel as the stationary phase for isolation of minor coumarins and furanocoumarins from the grapefruit oil prior to identification by gas chromatography–mass spectrometry (GC-MS) and NMR. These isolated compounds from flash column chromatography were identified as bergapten, 6′,7′-dihydroxybergamottin, auraptene, osthol, and polymethoxylated flavones.

Furanocoumarins extracted from fruits can be examined by HPLC or GC. Furanocoumarins are moderately volatile;

**Table 1 – Reverse-phase liquid chromatographic methods in the analysis of grapefruit furanocoumarins.**

| Sample | Eluent | Detector | Target analytes | Refs |
|---|---|---|---|---|
| Grapefruit oil | ACN & THF in water, methanol & THF in ACN | UV (310 nm), FL (Ex 310/Em 490 nm), & MS (SIM mode) | Bergapten, oxypeucedanin, epoxybergamottin, bergamottin | [39] |
| Grapefruit essential oil | ACN & THF in water, methanol & THF in ACN | UV (315 nm) | Bergamottin, epoxybergamottin, bergapten | [40] |
| Grapefruit essential oil, juice, & kernel | Water & ACN | UV (310 nm) & MS (MRM mode) | Bergapten & bergamottin | [41] |
| Grapefruit peel | 0.1% formic acid in water & methanol | MS (SIM mode) | Bergapten, 6′,7′-dihydroxybergamottin, epoxybergamottin, bergamottin | [42] |
| Different fractions of grapefruit juice | 0.05% formic acid in water and ACN | MS (SIM mode) | Aurapten, bergamottin, epoxybergamottin, 6′,7′-dihydroxybergamottin, furanocoumarin dimers | [43] |
| Grapefruit juice | Water & ACN | UV (310 nm) | Bergamottin, 6′,7′-dihydroxybergamottin, epoxybergamottin, furanocoumarin dimers | [44] |
| Grapefruit juice | 0.1% formic acid in water & ACN | UV (310 nm) | Bergaptol, psoralen, bergapten, 6′,7′-dihydroxybergamottin, bergamottin | [45] |
| Grapefruit juice | Water & methanol | UV (240 nm) | Paradisin A, paradisin B, bergamottin | [46] |

ACN = acetonitrile; FL = fluorescence; MS = mass spectrometry; MRM = multiple reaction monitoring; SIM = selected ion monitoring; THF = tetrahydrofuran.

thus, their contents can be analyzed by GC. However, some compounds have to be derivatized. The most common analytical method used for furanocoumarins identification is liquid chromatography (LC), which can analyze a wide range of furanocoumarins without derivitazation. Reverse-phase HPLC is typically used to quantify furanocoumarins in grapefruits. Previous studies indicate that the separation of the individual grapefruit furanocoumarins, including bergaptol, bergapten, bergamottin, expoxybergamottin, and 6′,7′-dihydroxybergamottin, can be achieved using reverse-phase HPLC columns [39,44,45]. Similarly, a total of 27 coumarins and furanocoumarins from citrus peel were isolated and identified simultaneously by a C18 reverse-phase column coupled with a mass spectrometer [42]. As for chromatography detectors, UV detection is most frequently used for quantification of furanocoumarins in grapefruits because of its simplicity and modest sensitivity. Results of UV spectra detected by the UV diode array detector indicate that bergapten, bergamottin, dihyroxybergamottin, and epoxybergamottin had the highest UV absorption at 310 nm [39]. Thus, several previous studies developed quantitative methods to analyze furanocoumarins in grapefruits by detection of the absorption at 310 nm wavelength (Table 1) [44,45,54]. The detection limit of UV absorption at 310 nm wavelength for furanocoumarins was 0.01–0.05 ppm (S/N = 8) [39]. In addition, fluorescence detection is also applicable for determination of furanocoumarins. The use of 310 nm wavelength in excitation and 490 nm wavelength emission provides good sensitivity for furanocoumarin detection. The detection limit of fluorescence for analyzing various furanocoumarins has been previously compared with UV detection [39]. Although the sensitivity of fluorescence detection is slightly higher than that of UV detection for certain furanocoumarins, others could not be detected at concentrations lower than 0.5 ppm. Because of its excellent sensitivity and accuracy, MS can give more accurate results for identifying and quantifying furanocoumarins. The detection limits of bergamottin were found to be 0.001 mg/L and 0.0005 mg/L for LC-ESI-MS/MS (LC-electrospray ionization-tandem MS) and LC-APCI-MS/MS (LC-atmospheric pressure chemical ionization-tandem MS), respectively, whereas UV detection was only 0.08 mg/L [41]. To distinguish the furanocoumarins coeluting from the HPLC column, Dugrand et al [42] have developed a rapid and sensitive LC-MS method that identified a total of six coumarins and 20 furanocoumarins using the single ion monitoring mode. Given that MS provides high sensitivity, selectivity, and accuracy for grapefruit furanocoumarin detection, this technology also can be applied to biological samples such as urine and plasma. Recently, a comprehensive ultraperformance liquid chromatography-MS/MS method has been established for determination of furanocoumarins in human plasma and urine after ingestion of grapefruit and grapefruit juice [55]. The identification, detection limit, quantitation limit, and recovery rate of furanocoumarins have been determined during this method validation. Bergamottin and 6′,7′-dihydroxybergamottin were identified as the major furanocoumarins found in plasma, whereas the predominant compounds detected in the urine were bergaptol and 6′,7′-dihydroxybergamottin.

## 4.    Distribution of furanocoumarins in grapefruit

Furanocoumarins in grapefruit all originate from psoralen, and the major grapefruit furanocoumarins include bergaptol, bergapten, bergamottin, epoxybergamottin, and 6′,7′-dihydroxybergamottin. The levels of furanocoumarins found in different grapefruit varieties have been determined by Girennavar et al [52]. Among the seven grapefruit varieties that include Rio Red, Ruby Red, Ray Red, Star Ruby, Thompson Pink, Marsh White, and Duncan, the concentrations of 6′,7′-dihydroxybergamottin, paradisin A, and bergamottin in grapefruit juice ranged from 0.5 μg/mL to 3 μg/mL, from 0.06 μg/mL to 0.08 μg/mL, and from 0.2 μg/mL to 1 μg/mL, respectively. Ray Red had the lowest levels of dihydrobergamottin and paradisin A, whereas the highest concentration of paradisin A was found in Star Ruby, and Duncan contained the highest amount of 6′,7′-dihydroxybergamottin and bergamottin. Furanocoumarins and their dimers have also been analyzed in commercial grapefruit juice. In a study comparing 28 white grapefruit juices, the average concentrations of bergamottin, GF-I-1 (paradisin A) and GF-I-4 (paradisin B) were 5.6 μg/mL, 0.32 μg/mL, and 0.96 μg/mL, respectively [46]. Similarly, Guo et al [12] analyzed the furanocoumarin monomers and dimers in seven grapefruit juice from local commercial sources. Their results showed that the concentrations of bergamottin, 6′,7′-dihydroxybergamottin, and epoxybergamottin ranging from 2.4 μg/mL to 10 μg/mL, from 0.3 μg/mL to 12.8 μg/mL, and from 0.17 μg/mL to 0.27 μg/mL, respectively, whereas the amounts of furanocoumarin dimers, paradisin A, B, and C, were relatively lower than bergamottin. A previous study also indicated that concentrations of furanocoumarins in white grapefruits were typically higher than those from red grapefruits [46,56]. Furanocoumarin concentrations in 29 white and 29 red commercial grapefruit juice from the retail market have been previously compared [44]. The average concentrations of bergamottin and epoxybergamottin in white grapefruit juices were 4.2 μg/mL and 2.9 μg/mL, respectively, whereas their contents in red grapefruit juice were 3.4 μg/mL and 1.8 μg/mL. Similar to results of furanocoumarin monomers, the concentrations of paradisin A and B in pink grapefruits were relatively lower than those in white grapefruits [46].

Variation of furanocoumarin distribution is also dependent on the different regions of grapefruit tissues. As reported in a previous study, concentrations of seven furanocoumarins from the peel, flesh, whole grapefruits, and grapefruit juice have been analyzed [55]. The highest concentrations of 6,7-dihydrobergamottin and bergamottin were 118.01 μg/g and 13.810 μg/g, and found in the peel and the flesh of the grapefruit, respectively. Interestingly, the investigation found that epoxybergamottin was only present in the peel and whole grapefruit, which indicates that epoxybergamottin originated solely in the grapefruit peel. Dugrand-Judek et al [2] also recently reported that the concentrations of epoxybergamottin in the peel of Duncan, Marsh, and Star Ruby varieties were much higher than those in the pulp. However, it is possible that epoxybergamottin is distributed to the juice or further hydrolyzed to 6′,7′-dihydroxybergamottin during the grapefruit juice manufacturing process [57]. In addition to the furanocoumarin monomers, the corresponding dimers located in different grapefruit sections have also been investigated [46]. Concentrations of bergamottin, paradisin A, and paradisin B found in grapefruit flesh were significantly higher than those in the peel. In contrast, these contractions in the juice sac and seeds were relatively low. Citrus essential oils extracted from plants consist of various constituents, specifically terpenes, alcohols, aldehydes, ketones, and esters. Because of their desirable aroma and health benefits, citrus essential oils are widely applied in the pharmaceutical, food, and fragrance industries. Along with the low-molecular-weight aroma components, some moderate volatiles, including coumarins and furanocoumarins, can also be extracted from the citrus peel or fruits during the manufacturing process of essential oil. Thus, several studies have developed different HPLC methods to measure the furanocoumarin concentrations in grapefruit essential oils. A previous study developed a HPLC method along with a UV detector to analyze 27 oxygen heterocyclic compounds in lemon, lime bergamot, grapefruit, mandarin, and bitter orange [40]. The average concentrations of bergapten and bergamottin in grapefruit essential oil were 263 mg/L and 1791 mg/L, respectively. Notably, grapefruit essential oil had the highest amount of epoxybergamottin among the seven essential oil samples. The quantitative HPLC methods for furanocoumarin concentrations in grapefruit essential oil by UV detector, fluorescence, or MS were compared in a previous study [39]. Although a UV detector has excellent precision at low levels, coelution of furanocoumarins may occur when analyzing essential oils. To avoid misleading peak identification, MS is a reliable technique for accurate quantification of furanocoumarins found in citrus oils. By using HPLC coupled with MS, the total concentration of furanocoumarins in the grapefruit oil was found to be 2236.7 μg/mL. Recently, the enantiomeric isomer of epoxybergamottin was also found in grapefruit oils using chiral column chromatography [58].

The formation of furanocoumarin in grapefruits can also be affected by environmental conditions. Temperature is a crucial factor affecting the composition of various nutrients and phytochemicals in citrus fruits. Storage of grapefruit juice at 100°C for 2 hours led to the loss of 6′,7′-dihydroxybergamottin and paradisin C, whereas amounts of bergaptol increased [59]. The effects of different storage temperatures on furanocoumarin levels in Rio Red and Marsh White grapefruit have also been discussed. After 30 days in storage, amounts of 6′,7′-dihydroxybergamottin, paradisin A, and bergamottin all decreased regardless of temperature (at 9°C or 24°C). However, the fruits stored at 9°C retained more furanocoumarins than those stored at 24°C. The influence of low storage temperatures on furanocoumarin levels has recently been reported [60]. The level of 6′,7′-dihydroxybergamottin in grapefruit stored at 2°C for 16 weeks was lower than in the fruits stored at 11°C for the same time point, whereas the bergamottin level significantly increased. This study also showed that low temperature conditioning carried out at 16°C over 7 days significantly reduced not only the incidence of chilling injury, but also the loss of bergamottin and expoxybergamottin. In addition to storage temperature, household processing techniques also significantly affect furanocoumarin levels in grapefruit juice. Juice processed using the blending technique had higher

concentrations of bergamottin, and hand-squeezed grapefruit juice resulted in higher concentrations of 6′,7′-dihydroxybergamottin when compared to the juicing technique [61]. Additionally, hand-squeezed grapefruit juice contained higher levels of 6′,7′-dihydroxybergamottin and bergamottin when compared to the commercially processed grapefruit juice. However, no significant differences were found in paradisin A concentrations [52]. The use of modified atmosphere packaging (MAP) is an effective method to not only maintain fruit quality such as flavor and texture, but also extend shelf life. Thus, a recent study investigated the influence of MAP on the constitutions of phytochemicals in Star Ruby grapefruits. After 16 weeks of storage, MAP significantly reduced the 6′,7′-dihydroxybergamottin level compared to the level in control fruits; yet ascorbic acid, limonoids, and fruit quality were not significantly changed [62]. Interestingly, the furanocoumarin concentrations in grapefruit juice were also affected by different container materials, such as cardboard, cartons, metal, and glass bottles [59,63].

## 5. Biological activities of furanocoumarins

### 5.1. Anticancer activity

Grapefruits are rich sources of vitamins, mineral elements, pectin, and other phytochemicals such as flavonoids, limonoids, and coumarins. These phytochemicals, consumed from the flesh of the fruits or derived products, are suggested to exhibit positive health benefits such as antioxidative, anti-inflammatory, antiproliferative, and neuroprotective activities [64,65]. Several studies have demonstrated that grapefruit juice exhibits chemopreventive and antigenotoxic activities both in vitro and in vivo [7,66–68]. For example, consumption of grapefruit juice over a 7-week period profoundly reduced azoxymethane-induced colon aberrant crypt formation, the earliest identifiable neoplastic lesion in the development of colorectal cancer, by elevating the antioxidant capacity [7]. A large number of studies have shown that several phytochemicals found in grapefruit, such as flavonoids and limonoids, had anticancer properties. Among these phytochemicals, naringin and hesperidin, two major flavonoids present in grapefruits, are recognized as the major bioactive components responsible for grapefruit's anticancer activities. Over the past few years, many in vitro and in vivo studies have suggested that in addition to flavonoids, grapefruit furanocoumarins also exhibit anticancer activities against numerous types of cancer including breast cancer, skin cancer, and leukemia.

Breast cancer is the most frequently occurring malignant neoplasm diagnosed in women worldwide and the second leading cause of cancer-related death among women in the United States [69]. In 2015, the American Cancer Society reported approximately 231,840 new cases of invasive breast cancer with 40,290 of these fatal diagnoses for United States women [70]. The typical treatment for breast cancer includes surgery, radiation therapy, and chemotherapy. Previous studies have suggested that phytochemicals isolated from citrus fruits exhibit promising potential for prevention or delay of the development of breast cancer [71–74].

Bergamottin, the major grapefruit furanocoumarin derived from psoralen, has been reported to exhibit an inhibitory effect on breast cancer cell growth. A recent study showed that treatment with bergamottin significantly suppressed the MDA-MB-231 breast cancer proliferation through inhibition of signal transducers and activator of transcription 3 (STAT3) expression [75]. When MDA-MB-231 cells were treated with bergamottin, phosphorylation and nuclear translocation of STAT3 were not only significantly reduced, but the binding activity of the STAT3 protein to the corresponding DNA sequence was also suppressed. STAT3 signaling is a major intrinsic pathway for cancer development because of its frequent activation in malignant cells, and also plays a key role in regulating several genes crucial for cancer inflammation. In the biosynthesis of furanocoumarins in grapefruits, both bergaptol and bergapten are precursor of bergamottin, and their inhibitory activities against breast cancer growth have also been recently studied. Panno et al [76] demonstrated that bergapten inhibited breast cancer cell growth through activation of p53 and caspases, suggesting that cell death resulted from apoptosis. In 2012, the same research group demonstrated that treatment with bergapten for 48 hours or 96 hours inhibited MCF-7 and ZR-75 breast cell proliferation in a dose-dependent manner [77]. The decrease in cell proliferation after treatment with bergapten was also found in tamoxifen-resistant MCF-7 cells. Tamoxifen is an antibreast cancer drug that competes with estrogen to bind the estrogen receptor once metabolized by cytochrome P450 enzymes. Surprisingly, bergapten reduced only estrogen receptor α protein expression, and its mRNA level was not affected. It was also confirmed that bergapten induced estrogen receptor depletion through SMAD4-mediated ubiquitination. Furthermore, bergapten can induce programmed cell death through both apoptosis, as well as autophagy. In MCF-7 and ZR-75 breast cancer cells, treatment with bergapten activated the expression of Beclin, UV radiation resistance-associated gene (UVRAG), and activating molecule in Beclin-1-regulated autophagy (AMBRA), and subsequently the autophagosomes, the evidence of autophagy, significantly increased [78]. The same study has also found phosphatase and tensin homologue deleted on chromosome 10 [phosphatase and tensin homologue (PTEN)] as the key target of bergapten and responsible for induction of breast cancer cell autophagy. Consistent with the role of bergapten against breast cancer growth through apoptosis, a recent study also reported that bergaptol significantly induced MCF-7 cell apoptosis through cleavage of caspase-9 and poly(ADP-ribose)polymerase as well as induced the expression of Bax and cytochrome c [79]. Also, it is noteworthy that aurapten, a major coumarin derived from umbelliferone in grapefruit, also exhibited its inhibitory activities against breast cancer growth in both in vitro and in vivo experiments [80,81]. Collectively, several grapefruit furanocoumarins possess breast cancer proliferation suppression potential through different molecular pathways.

Leukemia, malignant cancer of the blood cells, involves the production of abnormal white blood cell from the bone marrow. Multiple myeloma is a form of leukemia characterized by the accumulation of plasma cells in the bone marrow. Recently, the inhibitory activity of bergamottin against multiple myeloma cell growth has been studied [75]. Treatment

with bergamottin significantly reduced the proliferation of U266 cells in a time-dependent manner. The suppression of cell proliferation by bergamottin was mediated through the inactivation of phosphorylation of Janus-activated kinase (JAK) ½ and c-Src protein, followed by inhibition of STAT3 phosphorylation and nuclear translocation. STAT-3-regulated inflammatory and angiogenesis mediators, including cyclooxygenase-2 and vascular endothelial growth factor (VEGF), were also suppressed in a dose-dependent manner by bergamottin. The activation of STAT3 downregulated expression of antiapoptotic genes such as *Bcl-2*, *Bcl-xl*, *IAP-1*, and *survivin* suggests that bergamottin-induced cell death resulted from apoptosis. Furthermore, bergamottin in combination with simvastatin can enhance anticancer effects against human chronic myelogenous leukemia. The combination of these two compounds significantly potentiated tumor necrosis factor (TNF)-induced apoptosis through inactivation of nuclear factor κB (NF-κB) expression, as compared to the treatment with only an individual compound. Suppression of NF-κB further downregulated expression of cyclin D1, Bcl-2, Bcl-xL, VEGF, and matrix metallopeptidase 9 (MMP-9) [82].

Skin cancer originates from the uncontrolled growth of abnormal skin cells. In addition to exposure to UV radiation, the primary environmental cause of skin cancer, some chemicals and carcinogens can also initiate the development of skin carcinogenesis. In experimental mouse skin carcinogenesis models, benzo[α]pyrene (B[α]P) is commonly used to activate skin cancer initiation through reaction with DNA causing mutations at critical target genes responsible for skin carcinogenesis, specifically the Ras protooncogene [83]. Using five naturally occurring coumarins and furanocoumarins, Cai et al [84] demonstrated pretreatment with bergamottin to have the highest effects preventing covalent binding of B[α]P to mouse epidermal DNA. In a standard two-stage initiation–promotion experiment, bergamottin ameliorated the papilloma formation on the mouse skin initiated by application of B[α]P. The mechanism for delaying skin cancer development by bergamottin appears to involve inhibition of the cytochrome P450 enzymes responsible for the metabolic activation of B[α]P. Similarly, the same research group also found that pretreatment with bergamottin also blocked the DMBA-DNA adduct formation in a dose-dependent manner [85]. Bergamottin was further identified as a selective inhibitor of cytochrome P450 1A1, whereas imperatorin and isopimpinellin appear to selectively inhibit cytochrome P450 1B1. Previous studies also confirmed that bergamottin and 6′,7′-dihydroxybergamottin strongly inhibited cytochrome P450 3A [86,87].

In NCI-87 gastric carcinoma cells, bergamottin was found to exhibit the highest activity against CD74 expression among 25 different food phytochemicals, suggesting that bergamottin may serve as a potential candidate for treatment of *Helicobacter pylori* infection [88]. Bergamot essential oil, made from the fruit peel of the bergamot tree, contains considerable furanocoumarin concentrations, especially bergamottin. Recently, an *in vitro* study evaluated the anticancer activities of different components of bergamot oil against neuroblastoma cell growth. Results indicated that bergamot essential oil induced SH-SY5Y cell apoptosis through elevating reactive oxygen species production and regulating expression of mitogen-activated protein kinases (MAPKs) including p38 and

extracellular signal-regulated kinase (ERK1/2) and p53, Bcl-2, and Bax expression. These results suggest that the primary active compounds of bergamot essential oil that are responsible for anticancer activities in SH-SY5Y cells appear to be bergamottin and 5-geranyloxy-7-methoxycoumarin [89]. Bergamottin not only inhibits cancer cell growth, but also suppresses metastasis. Metastasis, the spread of cancer cells from one tissue to another away from the primary site of malignancy, is also the major cause of cancer-related death. In human fibrosarcoma cells, treatment with bergamottin potently reduced phorbol-12-myristate-13-acetate-induced MMP-9 and MMP-2 activation, subsequently suppressing cancer cell migration [90]. In metastasis, MMPs secreted by tumor cells destroy the extracellular matrix surrounding the tumor. Subsequently the tumor cells invade through the circulation of blood or lymph to facilitate the spread to distant tissues. In addition, this study found that reduction of MMP expression was upregulated by the inhibition of protein kinase C, p38, JNK phosphorylation, and NF-κB activation, all suggesting that bergamottin partly exerts anticancer activity through the suppression of MMP expression. The molecular mechanism of grapefruit furanocoumarins against cancer cell growth is summarized in Figure 2.

## 5.2. Antioxidative activity

A free radical is a molecule, atom, or ion that has one or more unpaired electrons. During pathogenesis of chronic diseases, overproduction of free radicals in the tissues causes oxidative damage to lipids, protein, and DNA because of its high reactivity toward other molecules. Thus, reduction of oxidative stress through scavenging free radicals is recognized as a promising strategy of phytochemicals to prevent or delay the occurrence of chronic diseases. The antioxidant activities of citrus limonoids, flavonoids, and coumarins have been compared in different *in vitro* experiments [91]. Although bergapten showed weaker antioxidative activities than flavonoids in a β-carotene-linoleic acid bleaching assay and superoxide radical scavenging activity assay, it did, however, offer some activities against copper-induced low-density lipoprotein oxidation in hamsters. It was also reported that the effect of bergapten to delay rat brain lipid peroxidation was similar to its precursor, psoralen [92]. The initiation time of bergapten for copper-mediated conjugated diene formation was comparable with that from naringin and its aglycone, naringenin. Bergaptol also exhibited free radical scavenging activities in 2,2′-azobis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazil (DPPH) assays in grapefruit [93]. Bergaptol isolated from the ethyl acetate extract of concentrated grapefruit juice scavenged > 75% free radicals in both assays, whereas the free radical scavenging activity of geranyl coumarin was relatively low. Comparison of 23 furanocoumarins isolated from *Angelica dahurica* revealed that bergaptol exhibited potent free radical scavenging activities in both DPPH and ABTS assays [94].

## 5.3. Anti-inflammatory activity

Inflammation is a complicated biological process in response to tissue injury, pathogens, or chemical stimulation. Chronic

inflammation is positively linked to several chronic diseases, such as cancer, cardiovascular disease, diabetes, and neurodegenerative disease [95]. In 2015, Uto et al [96] compared the anti-inflammatory activities of constituents isolated from either aerial or root parts of *Angelica acutiloba* using the lipopolysaccharide (LPS)-stimulated RAW 264.7 cell model. Using column chromatography, four compounds—tokiaerialide, Z-ligustilide, falcarindiol, and bergaptol—were identified. By comparison, bergaptol exhibited modest anti-inflammatory activities in RAW 264.7 cells through attenuation of the production of nitrite, prostaglandin E2, interleukin-6, and TNF-α. The anti-inflammatory activity of berpaptol originated, in part, from the upregulation of heme oxygenase-1 expression, which plays a protective role against inflammatory responses by elevating antioxidative activity. The biological activities of oxypeucedanin and oxypeucedanin hydrate, minor furanocoumarins present in grapefruits, have been reported [97]. Only oxypeucedanin effectively suppressed nitrite production and inducible nitric oxide synthase expression in LPS-activated RAW 264.7 cells, whereas the anti-inflammatory activity of oxypeucedanin hydrate was not significant.

## 5.4. Bone health promoting activity

Osteoporosis is a bone disease characterized by low bone mass and structural deterioration of bone tissue owing to an imbalance between bone resorption and formation. Osteoblasts are differentiated from mesenchymal stem cells and play a crucial role in creating, maintaining, and mineralizing skeletal architecture. These mononuclear cells are responsible for synthesizing bone matrix protein and maintaining acid—base homeostasis and calcium balance. Bergapten was found to be a bioactive furanocoumarin with antiosteoporosis activities in both *in vitro* and *in vivo* experiments. In 2004, Meng et al [98] investigated the proliferation-stimulating activities of different solvent fractions of crude extract of *Cnidium monnieri* on osteoblast-like UMR106 cells. The major bioactive compounds further identified were osthole, bergapten, and imperatorin. Bergapen significantly increased the cell proliferation percentage of UMR 106 cells by 24% as compared to the blank control group. In primary osteoblastic cells, bergaptol enhanced alkaline phosphatase (ALP) activity, type I collagen synthesis, bone nodule formation, as well as bone morphogenetic protein-2 (BMP-2) gene expression in a dose- and time-dependent manner [99]. Cotreatment with bergapten and noggin, an antagonist of BMP-2, attenuated the expression of ALP, indicating that upregulation of BMP-2 was involved in bone formation enhanced by bergapten. Bergapten was also found to enhance phosphorylation of SMAD 1/5/8, p38, and ERK. Pretreatment of osteoblastic cells with the MAPK inhibitors confirmed that the activation of p38 and ERK pathway is required for BMP-2 expression and maturation induced by bergapten. Furthermore, administration of bergapten for 7 consecutive days remarkably increased the bone volume and BMP-2 expression in the tibia of young rats. Collectively, this study suggests that BMP-2 was a key target for promoting bone formation by bergapten. Additionally, bergapten also promoted osteogenic differentiation in bone marrow stromal
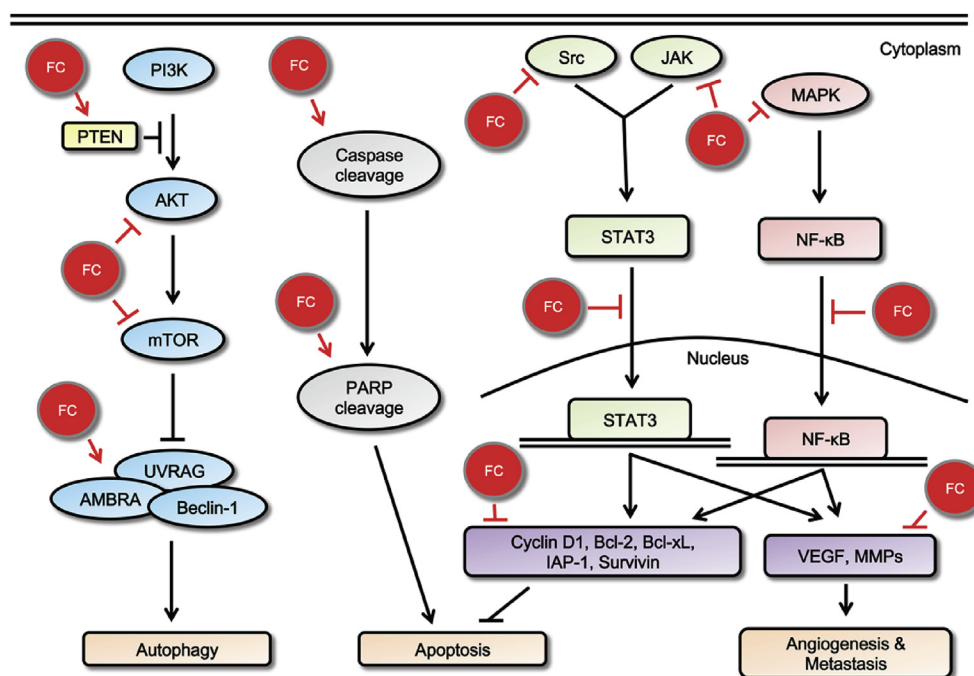


Figure 2 — **Proposed molecular mechanisms of grapefruit furanocoumarins against cancer cell growth.**
**FC = furanocoumarins; IAP-3 = inhibitor of apoptosis protein 3; JAK = Janus activated kinase; MAPK = mitogen-activated protein kinase; MMPs = matrix metallopeptidases; mTOR = mammalian target of rapamycin; NF-κB = nuclear factor-kappa B; PARP = poly(ADP-ribose) polymerase; PI3K = phosphoinositide 3-kinase; PTEN = phosphatase and tensin homologue; STAT3 = signal transducers and activators of transcription 3; VEGF = vascular endothelial growth factor.**

cells isolated from B6 mice [100]. Treatment with bergapten not only promoted ALP activation, but also upregulated the expression of Runt-related transcription factor 2 (RUNX2) and osteocalcin. Osteocalcin is the most abundant non-collagenous protein secreted by osteoblasts, and RUNX2 is a key transcription factor playing an important role in osteoblast differentiation [101,102]. Furthermore, the inhibitory activity of berapten on delaying bone loss was also studied in the ovariectomized (OVX) mouse model, the most popular animal model for studying postmenopausal osteoporosis. Oral administration of bergapten for 3 months effectively improved the bone mineral density, trabecular number, and trabecular separation compared to the OVX-alone treatment group. Along with the MAPK pathway activated by bergapten in osteoblastic cells, it was found that the Wnt/β-catenin pathway was also an important contributor to bone formation [99,100]. In addition to the promotion of osteoblastic bone formation, inhibition of osteoclastic bone resorption provides another feasible approach in the prevention of osteoporosis. Thus, a recent study investigated whether bergapten can promote bone health through the suppression of osteoblast growth [103]. This study showed bergapten reduced osteoclast formation in LPS-stimulated osteoblast precursor RAW 264.7 cells, as identified by tartrate-resistant acid phosphatase staining. Moreover, the cleavage of caspase-3 and -9 activated by bergapten suggests that growth of mature osteoclasts is suppressed through apoptosis. As several studies have demonstrated that consumption of grapefruit modulates bone strength in rats, bergapten may serve as the bioactive constituent in grapefruit to contribute bone health promoting activities [104–106].

## 6.    Concluding remarks

Furanocoumarins represent a subclass of polyphenolic compounds typically found in higher plants. The occurrence and distribution of furanocoumarins vary depending on citrus species. The major furanocoumarins found in grapefruit include bergamottin, 6′,7′-dihydroxybergamottin, and epoxybergamottin. All have been given extensive attention because of their undesirable interactions with certain medications. However, in the past few years, furanocoumarins have been found to possess positive biological activities including antioxidative, -inflammatory, and -cancer activities, as well as bone health enhancement. It should be noted that grapefruit furanocoumarins particularly exhibited potent anticancer activities against the growth of different types of cancer cells including skin cancer, breast cancer, leukemia, and neuroblastoma cells. Also, several molecular targets involved in the anticancer activities of grapefruit furanocoumarins have been found, such as upregulation of STAT3, NF-κB, PI3K/AKT, and MAPK expression. However, in vivo studies regarding the anticancer activities of furanocoumarins are still limited. In addition, different in vitro and in vivo studies have also demonstrated that furanocoumarins have anti-inflammatory and -oxidative activities and bone health promoting effects. Other biological activities of furanocoumarins such as neuroprotective activities or body weight regulations are still unclear. Thus, we suggest that further comprehensive research is required to confirm the health benefits of furanocoumarins as well as to investigate the related molecular mechanisms.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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