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Review

Antioxidant activity of *Citrus* fruits

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ABSTRACT

Citrus is well-known for its nutrition and health-promotion values. This reputation is derived from the studies on the biological functions of phytochemicals in *Citrus* fruits and their derived products in the past decades. In recent years, the antioxidant activity of *Citrus* fruits and their roles in the prevention and treatment of various human chronic and degenerative diseases have attracted more and more attention. *Citrus* fruits are suggested to be a good source of dietary antioxidants. To have a better understanding of the mechanism underlying the antioxidant activity of *Citrus* fruits, we reviewed a study on the antioxidant activity of the phytochemicals in *Citrus* fruits, introduced methods for antioxidant activity evaluation, discussed the factors which influence the antioxidant activity of *Citrus* fruits, and summarized the underlying mechanism of action. Some suggestions for future study were also presented.

Contents

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfpnic acid); APX, ascorbate peroxidase; As, arsenic; AuNPs, gold nanoparticles; B, boron; Ca, calcium; CAA, cellular antioxidant activity; CAT, catalase; CL, chemiluminescence; Co, cobalt; CO_3^{2-}, carbonate; COX, cyclooxygenase; CP, *Citrus* peels; CPT, carnitine palmitoyl transferase; Cr, chromium; Cu, copper; Cu^{2+}, copper ions; CUPRAC, cupric ion-reducing antioxidant capacity; db, dried base; DNAG, deacetylation millington acid 17-β-D-glucoside; DPPH, 1,1-diphenyl-2-pierylhydrazy; DW, dry weight; Fe, iron; Fe^{2+}, ferrous ions; FRAP, ferric reducing-antioxidant power; Ge, germanium; GGT, γ-glutamyltranspeptidase activity; GPx, glutathione peroxidase; G6PD, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; GSH, reduced glutathione; GSHPx, glutathione peroxidase; GSt, glutathione-S-transferase; HO-1, heme oxigenase-1; H_2O_2, hydrogen peroxide; HPLC, high performance liquid chromatography; HPLC-FRSD, high performance liquid chromatography-free radical scavenging detection; HOCl, hypochlorus acid; K, potassium; LDL, oxidation of low density lipoprotein; LG, limonin 17-β-D-glucoside; LOX, lipoxygenase; LPO, lipid peroxidation; MDA, malondialdehyde; Mg, magnesium; Mn, manganese; Mo, molybdenum; MPO, myeloperoxidase; MPR, myoglobin protective ratio; Na, sodium; NAG, millington acid 17-β-D-glucoside; NF-κB, nuclear factor kappa B; Ni, nickel; NO, nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidase; NPs, nanoparticles; NQO, NADPH-quinone oxidase; Nrf-2, nuclear factor E2-related protein 2; O^{2-}, superoxide anion; $^1O_2$, singlet oxygen; OH, hydroxyl radical; OG, obacunone 17-β-D-glucoside body; $ONOO^-$, eroxynitrite; ORAC, oxygen radical absorbance capacity; PAL, phenylalanine ammonia lyase; P, phosphorus; PCL, photochemiluminescence; POD, peroxidase; PON, paraoxonase; PPO, polyphenoloxidase; RNS, reactive nitrogen species; ROO, peroxyl radical; ROS, reactive oxygen species; S, sulfur; Se, selenium; Si, silicon; SNPAC, silver nanoparticle antioxidant capacity; SOD, superoxide dismutase; SOD_2, mitochondrial superoxide dismutase; TAC, total antioxidant capacity; TEAC, Trolox equivalent antioxidant capacity; TRAP, total radical-trapping antioxidant parameter; XO, xanthine oxidase; Zn, zinc.

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1. Introduction

Citrus, the genus *Citrus* L. of the family Rutaceae, is one of the most important fruit crops in the world. It is widely grown in the tropical and subtropical areas of the world, and many other areas, with an annual production of approximately 102 million tons (Mehl et al., 2014). *Citrus* fruits are well-accepted by consumers of all over the world because of their attractive colours, pleasant flavors and aroma. Along with an increase of production, advances in storage and processing techniques, and the realization of a year-round supply, *Citrus* fruits have now become an important dietary source of nutrients for Chinese people.

Antioxidant activity denotes the ability of a bioactive compound to maintain cell structure and function by effectively clearing free radicals, inhibiting lipid peroxidation reactions, and preventing other oxidative damage (Bravo, 1998). It is also a foundation of many other biological functions, such as anti-cancers, anti-inflammation and anti-aging (Cai, Luo, Sun, & Corke, 2004; Ke, Pan, Xu, Nie, & Zhou, 2015). More importantly, the prevention of many chronic diseases, such as cancer, diabetes and cardiovascular disease, has been suggested to be associated with the antioxidant activity (Rajendran et al., 2014; Yu et al., 2005). Therefore, a deep study of natural antioxidants, such as those from fruits and vegetables, is of great importance to human health.

Citrus fruits are rich sources of useful phytochemicals, such as vitamins A, C and E, mineral elements, flavonoids, coumarins, limonoids, carotenoids, pectins, and other compounds (Zhou, 2012). These phytochemicals, consumed through fresh fruits or their derived products, have been suggested to have a wide variety of biological functions including antioxidant, antiinflammation, antimutagenicity, anticarcinogenicity and anti-aging to human health (Ke et al., 2015; Rajendran et al., 2014; Zhang et al., 2015).

To provide a comprehensive view of current studies on the antioxidant activity of *Citrus* fruits, the antioxidant components and their antioxidant activities, evaluation methods, and the internal and external factors that influence the antioxidant capacity of *Citrus* fruits were systematically reviewed. Most importantly, the mechanisms of antioxidant action of *Citrus* fruits were summarized for the first time.

2. The antioxidant components of *Citrus* fruits and their antioxidant activities

2.1. Vitamins in Citrus fruits and their antioxidant activities

There are more than 170 antioxidants from *Citrus* fruits that have been reported in the current literature, including vitamins, mineral elements, phenolic compounds, terpenoids and pectin (Zhou, 2012). Table 1 summarizes their representative types, chemical structures and antioxidant properties.

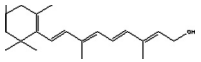
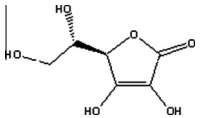
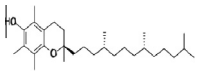
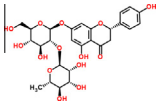
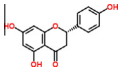
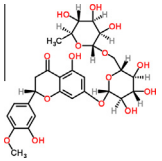
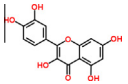
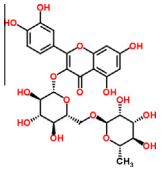
Vitamins are organic substances vital for body function and indispensable to our life. Among the 13 vitamins reported in literature, six of them are found in *Citrus* fruits, including vitamin A, vitamin B1, vitamin B2, vitamin C, vitamin E and vitamin B3 (Zhou, 2012). Of these vitamins, vitamin A, vitamin C and vitamin E were evaluated for their antioxidant activities (Amitava & Kimberly, 2014).

Vitamin A is a class of fat-soluble organic compounds, which includes retinol, retinal, retinoic acid, and several provitamin A (β-carotene) (Amitava & Kimberly, 2014). *Citrus* fruits are rich in carotenes and cryptoxanthin. The content of vitamin A in candied orange (*Citrus sinensis* Osbeck) is 0.27 mg/kg, and 2.77 mg/kg in tangerine (*Citrus reticulata* Blanco.) (Zhou, 2012). According to the reports, vitamin A can react with free radicals (especially singlet oxygen ($^1O_2$)) and peroxyl radicals to show its antioxidant property (Table 1).

Vitamin C, L-ascorbic acid or simply ascorbate, is a water-solubility substance. It is a major vitamin found in *Citrus* and rich in the flesh and peel of fruits. The content of vitamin C in the extraction of satsuma mandarin (*Citrus unshiu* Marc.), pomelo (*Citrus maxima* (Burm Merr.), navel orange (*C. sinensis* Osbeck), and tangerine (*C. reticulata* Blanco.) reached 30–40 mg/100 g, 60 mg/100 g, and 30 mg/100 g, respectively (Ye, 2005). Vitamin C is a natural free radical scavenger, which can effectively scavenge a variety species of reactive oxygen species (ROS) and give off semi dehydroascorbic acid, clearing $^1O_2$ and reducing sulfur radicals (Table 1, Amitava & Kimberly, 2014).

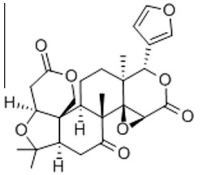
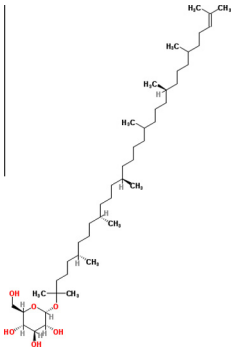
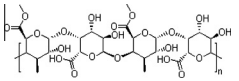
Vitamin E, another fat-soluble vitamin, is a group of compounds that include both tocopherols and tocotrienols. Vitamin E is mainly

Table 1
Antioxidants in *Citrus* fruits, their chemical structures and antioxidant activity reported in current literature.

Compound name			Basic structure	Antioxidant activity	Key references
Vitamins	V_A			(i) React with free radicals, especially $^1O_2$ (ii) React with peroxyl radicals	Amitava and Kimberly (2014)
	V_C			(i) Scavenge variety species of ROS (ii) Give off Semi dehydroascorbic acid (iii) Clear $^1O_2$ (iv) Reduce sulfur radicals	Amitava and Kimberly (2014)
	V_E			(i) Restrain free radicals and quenches $^1O_2$ (ii) Reduce ferrous iron to ferric iron to minimize catalysis (iii) Synergistic effect with selenium to protect mitochondria against free radical damage and their membranes against peroxidation damage (iv) Prevent the oxidation of carotenoid, enhancing their antioxidant capacity	Levander et al. (1995) and Amitava and Kimberly (2014)
Mineral elements	Selenium Zinc Copper Iron Manganese		Se=Se Zn Cu Fe Mn	(i) Se: Destroy free radicals in the cytoplasm as an essential component of antioxidant enzyme glutathione peroxidase (GSH-Px) (ii) Se: Synergistic effect with vitamin E to protect mitochondria against free radical damage and their membranes against peroxidation damage	Levander et al. (1995), Amitava and Kimberly (2014)
Phenolic compounds	Flavonoids	Naringin	 	(i) Inhibit the body's oxidant enzymes (ii) Improve the body's antioxidant enzyme activity (iii) Scavenge ROS directly (iv) Anti-lipid oxidation *in vitro* (v) Decrease quality of peroxide formation *in vivo*	Nakao et al. (2011)
		Naringenin			
		Hesperidin			
		Quercetin			
		Rutin			
	Phenolic acid			(i) Have different levels of free radical scavenging effect (ii) The dehydrogenation capacity of hydroxyl group (iii) The effect of ortho substitution on benzene ring	Dai and Mumper (2010)
	Coumarins			(i) Coumarins have been shown to possess strongly antioxidant activities because of their phenolic hydroxyl groups in molecule structure (ii) Direct decrease the cellular free radical production by inhibiting XO	Tyagi et al. (2005), Lin et al. (2008)

(*continued on next page*)

Table 1 (*continued*)

Compound name		Basic structure	Antioxidant activity	Key references
Terpenoids	Limonoids		(i) Induce apoptosis and free radical (ii) Different limonoids have variable antioxidant capacity	Poulose et al. (2005)
	Carotenoids		(i) Quenching $^1O_2$ (ii) Eliminate harmful free radicals	Di Mascio et al. (1989)
Pectin			(i) Enhance endogenous antioxidant enzymes (ii) Disposal of free radicals	Koriem et al. (2014)

found in the peels and seeds of *Citrus* fruits, and its content in candied orange (*C.* sinensis Osbeck), tangerine (*C. reticulata* Blanco.) and lemon (*Citrus limon* Burm.f.) can reach 5.60 mg/kg, 4.50 mg/kg, and 11.40 mg/kg, respectively (Zhou, 2012). Vitamin E can protect cell membranes against lipid peroxidation damage through many different ways, and the details are given in Table 1.

2.2. Mineral elements in Citrus fruits and their antioxidant activities

Mineral elements are the chemical compounds that plants take from the soil for their growth and development with the exception of carbon, hydrogen and oxygen (Zhou, 2012). Among the 81 chemical elements found in mammalian bodies, at least 19 are found in *Citrus* plants, including calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K), sulfur (S), sodium (Na), iron (Fe), manganese (Mn), nickel (Ni), boron (B), silicon (Si), copper (Cu), zinc (Zn), molybdenum (Mo), selenium (Se), cobalt (Co), chromium (Cr), germanium (Ge) and arsenic (As) (Zhou, 2012). Of these elements, Mn, Fe, Cu, Zn and Se have been reported to be related to the antioxidant activity of organisms (Table 1, Amitava & Kimberly, 2014). For example, Se, an essential component of antioxidant enzyme GSH-Px, can destroy free radicals in the cytoplasm and protect the tissues against oxidative damage (Levander, Ager, & Beck, 1995). The content of Se in the extraction of candied orange (*C. sinensis* Osbeck), tangerine (*C. reticulata* Blanco.), and lemon (*C. limon* Burm.f.) are 0.31 μg/100 g, 0.45 μg/100 g, and 0.50 μg/100 g, respectively (Zhou, 2012).

2.3. Phenolic compounds in Citrus fruits and their antioxidant activities

2.3.1. General

Polyphenols comprise a variety of bioactive compounds that are commonly divided into several classes, hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanidins, flavanoids, stilbenes and lignans (Zhou, 2012). Among the *Citrus* fruits phenolic compounds, the antioxidant activity of flavonoids, phenolic acid, and coumarins are the most studied in existing literature.

2.3.2. Flavonoids

Flavonoids have a direct role in scavenging reactive oxygen species, which can counteract lipid oxidation *in vitro* and improve the body's antioxidant enzyme activity, and decrease peroxide formation *in vivo* (Nakao et al., 2011). Among the *Citrus* flavonoids, the antioxidanty of naringin, hesperidin and naringenin are commonly studied.

Naringin can significantly enhance the immune system's effectiveness to avoid internal organs and tissue injury or disease caused by oxidation by increasing the activity of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), paraoxonase (PON) and other antioxidant enzymes (Ali & El Kader, 2004). The content of naringin in candied shaddock (*Citrus grandis* (L.) Osbeck) can reach 11.90 mg/100 g (Zhou, 2012).

Hesperidin has DPPH scavenging ability and it can dose – dependently inhibit the Cu^{2+}-induced oxidation of low density lipoprotein (LDL) *in vitro*, promote pancreatic B cells regeneration, and prevent the oxidative stress on the embryos of diabetic pregnant rats (Toumi et al., 2009). The content of hesperidin in candied orange (*C. sinensis* Osbeck), lemon (*C. limon* Burm.f.) and shaddock (*C. grandis* Osbeck) is 11 mg/100 g, 22 mg/100 g, and 4.37 mg/100 g, respectively (Zhou, 2012).

Naringenin can inhibit the β-oxidation of fatty acids in the liver by regulating the enzymes of the fatty acid oxidation processes, such as carnitine palmitoyl transferase (CPT, the rate-limiting enzyme of fatty acid oxidation), 3-hydroxy-3-methyl-glutaryll-CoA reductase, PON and plasma antioxidant enzymes (Jung, Lee, Park, Kang, & Choi, 2006). Naringenins are more effective than hesperidin (Jung et al., 2006). The content of naringenin can be 75 mg/l in Sunkist grapefruits (Ho, Saville, Coville, & Wanwimolruk, 2000).

In addition, other flavonoids, such as quercetin and rutin, are reported to be able to scavenge hydroxyl radicals (Table 1) (Terao & Piskula, 1998). The amounts of quercetin in shaddock (*C. grandis* Osbeck) can reach 24.09 mg/100 g (Zhou, 2012).

2.3.3. Phenolic acids

The phenolic acids are rich in *Citrus* fruits and have different levels of free radical scavenging. *Citrus* phenolic acids show strong antioxidant properties through the dehydrogenation of hydroxyl groups and the effect of ortho – substitution on a benzene ring (Table 1) (Dai & Mumper, 2010). The total content of *Citrus* phenolic acids ranged from 180 mg/g dry weight (DW) in flesh of Huyou (*Citrus paradisi* Macf. Changshanhuyou) to 5060 mg/g DW in peel of Ponkan (*Citrus poonensis* Hort. ex Tanaka) (Xu, Ye, Liu, Ma, & Chen, 2008).

2.3.4. Coumarins

Coumarins are another class of phenolic compound rich in *Citrus* fruits. They are derived from a branch of the phenylalanine metabolism pathway, which leads ultimately to furanocoumarin (psoralin) synthesis (Yu et al., 2005). Coumarins have been shown to possess strong antioxidant activities because of their phenolic hydroxyl groups (Tyagi et al., 2005). The content of coumarins in shaddock (*C. grandis* Osbeck) is about 2.2 mg/100 g (Zhou, 2012).

2.4. Terpenoids in Citrus fruits and their antioxidant activities

2.4.1. General

Among the *Citrus* terpenoids, the antioxidant activities of limonoids (Poulose, Harris, & Patil, 2005) and carotenoids (Di Mascio, Kaiser, & Sies, 1989) are reported in Table 1.

2.4.2. Limonoids

Limonoids are a group of highly oxygenated, tetracyclic triterpene secondary metabolite derivatives (Yu et al., 2005). 36 limonoid aglycones and 17 limonoid glycosides have been reported in *Citrus* fruits (Zhou, 2012). Different limonoids have variable antioxidant capacities and some are even better than vitamin C. For example, the free radical scavenging activity of four limonin glycosides, including limonin 17-β-D-glucoside (LG), obacunone 17-β-D-glucoside body (OG), millington acid 17-β-D-glucoside (NAG) and deacetylation millington acid 17-β-D-glucoside (DNAG), were evaluated, and it was found that all of them have antioxidant activities, among which NAG is the strongest and LG is the weakest (Poulose et al., 2005). The content of limonoids in *Citrus* fruits ranges from 0 to 95.46 mg/100 g (Zhou, 2012).

2.4.3. Carotenoids

Carotenoids are a type of tetraterpenoids. They have been reported to show antioxidant activities through quenching $^1O_2$ and eliminating harmful free radicals (Di Mascio et al., 1989). They may also protect immune cell membrane lipids from oxidative damage, thus ensuring communication signals between cells and receptors on the cell membrane to maintain normal cell function and enhance human immunity (Zhou, 2012). The total content of carotenoids in the peels of ponkan (*C. reticulata* Blanco), Wendun (*C. grandis* Osbeck) and Peiyou (*C. grandis* Osbeck) are 2.04 ± 0.036 mg/g dried base (db), 0.036 ± 0.0006 mg/g db and 0.021 ± 0.0004 mg/g db, respectively (Wang, Chuang, & Hsu, 2008).

2.5. Pectin in Citrus fruits and its antioxidant activity

Pectin is the major component of the cell wall in plants. It is chemically a polysaccharide, consisting of a linear chain of linked galacturonic acid. It has been reported that pectin decreased blood lipid level and peroxidative status, and showed antioxidant activities in kidney toxicity induced by octylphenol (Koriem, Arbid, & Emam, 2014). The suppressive effect of pectin appears to be through enhancement of endogenous antioxidant enzymes and disposal of free radicals (Table 1); where pectin at a higher dose was more potent (Koriem et al., 2014). In *Citrus* fruits, pomelo and lemon usually have a higher pectin content, and their total pectin contents range from 36.0 ± 1.46 mg/g db to 86.4 ± 3.36 mg/g db in the peels of *Citrus* fruits cultivated in Taiwan (Wang, Chuang et al., 2008).

2.6. Contribution of different antioxidant components of Citrus fruits to their total antioxidant capacity

The total antioxidant capacity of plant extracts is influenced by their chemical composition and content of antioxidants. Different components in plant extracts contribute unequally to their total antioxidant ability. In a lipid peroxidation system, the antioxidant capacity of different *Citrus* fruit extracts are positively associated with their total polyphenol content, while no obvious correlation was found with their vitamin C content. This result might suggest that *Citrus* polyphenols may be the dominant antioxidant component (Franke et al., 2004). However, Del Caro, Piga, Vacca, and Agabbio (2004) found that the antioxidant capacity of *Citrus* fruits of different species and cultivars was clearly correlated with the ascorbic acid content rather than with the presence of flavanone glycosides. Moreover, Rekha et al. (2012) found that the antioxidant capacity of fruit juices was directly related to the content of total phenolics and vitamin C in some *Citrus* species. Furthermore, Wu, Peng, and Peng (2009) reported that a high correlation was found between the scavenging activity and the flavonoid content in *Citrus* branches. A comparison of the antioxidant activity of different *Citrus* components is needed in the future.

3. Methods for the antioxidant activity evaluation of *Citrus* fruits

3.1. General

Nowadays, the antioxidant capacity of plant foods has been taken as an indicator of their beneficial effects on human health (Prior & Wu, 2013). Therefore, a good method for the antioxidant activity evaluation of *Citrus* fruits is of great importance. There are a wide variety of antioxidant activity evaluation methods for plant samples that have been reported in the existing literature. To present a general view of the potential methods that can be used for the antioxidant activity evaluation of *Citrus* fruits, we introduce the *in vitro* and *in vivo* assays, HPLC-based on-line methods, nanoparticle-based as well as myoglobin-based methods. Where possible, the application of these methods to *Citrus* fruits is presented.

3.2. In vitro assays

In vitro assays, also called chemical methods, are mainly concerned with the free radical or oxide scavenging capacity of the antioxidants. In the current literature, the major *in vitro* methods used for plant samples are 1,1-diphenyl-2-pierylhydrazy (DPPH) (Alam, Bristi, & Rafiquzzaman, 2013), 2,2′-azino-bis(3-ethylbenz thiazoline-6-sulfpnic acid) (ABTS) (López-Alarcón & Denicola, 2013), oxygen radical absorbance capacity (ORAC) (López-Alarcón & Denicola, 2013), total radical-trapping antioxidant parameter (TRAP) (Alam et al., 2013), trolox equivalent antioxidant capacity (TEAC) (Netzel, Netzel, Tian, Schwartz, & Konczak, 2007), ferric reducing-antioxidant power (FRAP) (Alam et al., 2013), cupric ion-reducing antioxidant capacity (CUPRAC) (Alam et al., 2013), and photochemiluminescence (PCL) (Netzel et al., 2007). The principal advantage of these methods is speed and simplicity, but the disadvantage is that their results are influenced by many factors, such as antioxidants and interactions, interference materials, pH, action time, producing system for free radicals and so on. In respect to *Citrus* fruits, the DPPH, FRAP, ABTS, and ORAC methods are often used to evaluate antioxidant activity (Del Caro et al., 2004; Rekha et al., 2012; Sánchez-Moreno et al., 2005; Wolfe et al., 2008; Xi, Fang, Zhao, Jiao, & Zhou, 2014; Zhang et al., 2014).

3.3. In vivo assays

For all *in vivo* methods, samples to be tested are administered to the testing animals (rats, mice, etc.) at a definite dosage regimen, usually as described by the respective method. After a specified period of time, the testing animals are sacrificed and tissues or blood are used for the assay (Alam et al., 2013). The *in vivo* methods reported in the existing literature for the antioxidant activity evaluation of plant samples include enzyme activity assays (Alam et al., 2013) and cellular antioxidant activity (CAA) assays (Wolfe et al., 2008).

Enzymes involved in *in vivo* metabolism are important to maintain cellular redox status (López-Alarcón & Denicola, 2013). In the current literature, the major enzyme activity assays used for plant samples are reduced glutathione (GSH), glutathione peroxidase (GSHPx) glutathione-S-transferase (GSt), SOD, CAT, γ-glutamyltranspeptidase activity (GGT), glutathione reductase (GR), LDL, and lipid peroxidation (LPO) (Alam et al., 2013).

The CAA assay was developed by Wolfe et al. (2008). Due to the ethical issues with human study and the time-consuming and expensive characters of animal experiments, this method is widely recognized as a fairly reliable method for the antioxidant activity evaluation of phytochemicals and food extracts. So far the commonly used cell models for evaluating antioxidant activities of phytochemicals are Caco-2, HepG-2 (Quéguineur et al., 2012), and MCF-7 (Yang & Liu, 2009). However, no report on *Citrus* fruits was found in the current literature.

3.4. On-line methods based on HPLC system

Traditional off-line methods for the antioxidant activity evaluation of plant samples have several problems, mainly time-consuming and high labor intensity (Zhang et al., 2015). On the other hand, the on-line methods based on HPLC, including on-line HPLC-chemiluminescence (CL) detection (Hartkopf & Delumyea, 1974), HPLC-DPPH method (Bandoniene & Murkovic, 2002), on-line HPLC-ABTS method (He et al., 2010), and on-line HPLC-CUPRAC method (Çelik, Özyürek, Güçlü, & Apak, 2010), can detect the antioxidant activity of a single constituent and its contribution to the overall activity of complex mixtures simultaneously. The antioxidant activity of a single constituent can then be compared with others in the complex matrix (Esin Çelik, Özyürek, Güçlü, Çapanoğlu, & Apak, 2014). Therefore the purification of every single compound for off-line assays are no longer required, leading to a very significant reduction of time and cost to obtain results.

In the existing literature, many different on-line methods have been demonstrated to be a useful screening tool for investigating antioxidants in plants. For example, Ou, Schmierer, Rades, Larsen, and McDowell (2013) used an on-line HPLC–DPPH assay system to identify the key antioxidants in leaf extracts of Sonchusoler-

aceus and to investigate the effect of extraction conditions and leaf position on the antioxidant activity and concentration. The study found upcaftaric acid, chicoric acid and chlorogenic acid as the key antioxidants identified in the leaf extracts.

To evaluate the antioxidant activity of *Citrus* fruits, our group developed an on-line high performance liquid chromatography-free radical scavenging detection (HPLC-FRSD) system for the total antioxidant capacity evaluation of *Citrus* fruits (Zhang et al., 2015). Based on the free radical decolorisation assays, which examine the capacity of an antioxidant to scavenge the free radicals ABTS or DPPH, our HPLC-FRSD system can rapidly detect the antioxidants and their antioxidant activity of a sample in a post-column instrument. Using the on-line HPLC-FRSD system, the antioxidant activities of 35 *Citrus* genotypes were studied (Zhang et al., 2015).

3.5. Myoglobin-based method

Myoglobin is a novel fluorescence probe. Terashima, Nakatani, Harima, Nakamura, and Shiiba (2007) proposed a myoglobin-based antioxidant activity evaluation method, which measured the antioxidant activity of samples by examining the change in the fluorescence absorbance, which resulted from the reaction of myoglobin with ROS. The myoglobin protective ratio (MPR) was defined to express the antioxidant levels of the specimens tested (Terashima et al., 2012). The method was suggested to be rapid, simple and reliable. Currently, it is used to evaluate the antioxidant activity of cooked Gomchwi (*Ligularia fischeri*) (An, Park, & Kim, 2014).

3.6. Nanoparticle-based method

Novel chemical assays based on nanotechnology, particularly using nanoparticles, have been proposed to evaluate antioxidant activities (Scampicchio et al., 2006). This approach is generally based on the reduction of metal complexes by natural antioxidants to generate NPs. In a pioneering study, Özyürek, Güngör, Baki, Güçlü, and Apak (2012) developed a silver nanoparticle antioxidant capacity (SNPAC) method for the antioxidant activity evaluation of common food. Their method employs Trolox as standard antioxidant to reflect the total antioxidant capacity (TAC) of the sample. As the first step, silver seeds were formed by reducing Ag^+ ions with citrate. Then, to increase the plasmon absorption intensity, the polyphenol-containing samples were added. Polyphenols acting as secondary reducing agents provided a more robust and reproducible method than those employing metal ions by direct reduction by antioxidants. The advantage of this method is the good linearity with polyphenol concentration, which is not affected by the presence of fruits acids, reducing sugars, or amino acids in the extracts.

Most recently, a new antioxidant activity assay using gold nanoparticles (AuNPs) was developed (Vilela, Castañeda, González, Mendoza, & Escarpa, 2015). In this method, the formation of AuNPs will depend on the total reduction contribution of all complexes involved in the plant extracts, therefore, their overall antioxidant action was evaluated by examining the presence of polyphenols (Vilela et al., 2015). The method was also suggested to be rapid, inexpensive and reliable. Although nanoparticle-based methods open a promising field for the antioxidant activity evaluation of natural products, until now, the practical application of these methods is rare.

4. Factors influencing the antioxidant capacity of *Citrus* fruits and products

4.1. General

The antioxidant system of a biological sample is complicated and might be influenced by a wide array of factors. The phytochemicals of *Citrus* plants are diverse and vary with its species, origin, and different tissues. Therefore, their antioxidant capabilities are also different. For simplicity of description, the internal and external factors that influence the antioxidant capacity of *Citrus* fruits and their derived products are divided into the following categories: the chemical structure of the antioxidants, pre- and post-harvest factors, and processing factors.

4.2. The chemical structure of Citrus antioxidants and their activities

The activity of antioxidants is closely related to their chemical structure. Among the antioxidants reported in *Citrus* fruits, flavonoids, coumarins, limonoids, and phenolic acids are reported to have structure–activity relationships (Table 1).

The structure of flavonoids and their relationship to antioxidant activity have been elaborated in many studies. The glycosylation of flavonoid compounds usually decreased their antioxidant capacity. For example, narigin compared with its aglycon-form naringenin usually has a lower radical scavenging efficiency (Kim & Lee, 2004). The hydroxyl structure on the B ring usually influences the radical scavenging efficiency of flavonoids or its derivatives, and the hydroxyl number increases such activities (Wang & Joseph, 1999; Yu et al., 2005). In addition, a lower antioxidant activity of limonoids than other flavonoids was considered to be due to the lack of pigmented rings in limonoids (Yu et al., 2005).

In spite of the fact that bound phenolic acids possessed higher antioxidant activities, analysis of antioxidant potentials and their relationship with phenolic acid content showed that free phenolics were more effective (Dai & Mumper, 2010).

The pharmacodynamics effect of coumarin is associated with substituent species and their positions on the parent nucleus (Hoult & Paya, 1996). Orthodihydroxy and orthohydroxy-amino coumarins were found to possess the highest antioxidant and radical scavenging activities (Tyagi et al., 2005).

As the number of bioactive compounds identified from *Citrus* fruits increases, the structure–activity relationships of the other *Citrus* bioactive compounds should be paid more attention.

4.3. Pre-harvest factors

The pre-harvest factors that influence the antioxidant activity of *Citrus* fruits include environmental conditions and agronomic conditions, which act by affecting the level of phytochemicals (Tiwari & Cummins, 2013).

The environmental conditions, such as soil moisture, temperature variation, sunshine radiation, and climatic conditions within a geographical location, influence the level of antioxidants in *Citrus* fruits. Research has shown that, the type and content of *Citrus* phytochemicals varies with soil type and depth. Temperature difference between day and night has obvious effects on the content of flavonoids, phenolic acid and anthocyanin, thereby affecting the antioxidant capacity of *Citrus* fruits (Zheng & Wang, 2001). In the growth period, the content of nutrients and bioactive compounds goes together with the solar radiation and hours of sun. Dry or over wet soil both cause a decrease in contents of bioactive compounds (Lund & White, 1986).

The agronomic conditions including fertilizer, sowing date, irrigation, maturity stages and subsequent harvesting, influence the level of antioxidants in *Citrus* fruits. It is worth mentioning that the physiological maturity of *Citrus* fruits plays a key role in the level of phytochemicals. Harvesting *Citrus* fruits at an over-ripened stage causes a higher phenolic and anthocyanins content than those harvested at un-ripened stages (Pantelidis, Vasilakakis, Manganaris, & Diamantidis, 2007). Moreover, there exist other agronomic factors, such as oxalic acid treatment, salicylic acid treatment and calcium treatment (Martínez-Esplá

et al., 2014), which also influence the antioxidant activity of *Citrus* fruits. Yet other results suggest that fruits grown organically contain higher levels of total phenolics and anthocyanin compared to those grown conventionally (Wang, Chen, Sciarappa, Wang, & Camp, 2008).

4.4. Post-harvest factors

Fruit harvesting and post-harvest treatment is the main factor that affects antioxidant activity. The post-harvest factors that influence the antioxidant capacity of *Citrus* fruits mainly includes storage conditions, such as time, temperature, humidity, light intensity and agro-chemicals.

Many studies indicated that proper storage conditions produce a raise of bioactive compounds in most fruits, further improving their antioxidant capacities. Fruits have an increased ability to induce flavonoid accumulation (especially anthocyanins) during cold storage (Chaudhary, Jayaprakasha, Porat, & Patil, 2014). For example, in blood oranges under low temperature storage, there is an increase in flavanones, anthocyanins and hydroxycinnamic acids and a mildly decrease in vitamin C (Rapisarda, Bianco, Pannuzzo, & Timpanaro, 2008).

Gas composition has a strong influence on the antioxidant activity of fruits, which means the concentrations of carbon dioxide and oxygen need to be controlled appropriately (Moretti, Mattos, Calbo, & Sargent, 2010). Studies have shown that under a high O_2 atmosphere, the higher storage quality delivers higher total phenolic content and DPPH· scavenging capacity (Yang, Zheng, & Cao, 2008).

Ethylene, an important natural plant hormone used in agriculture to promote the ripening of fruits, plays an important role in stimulate activity of phenylalanine ammonia lyase (PAL), a key enzyme in the biosynthesis of phenolic compounds and accumulation of phenolic constituents (Oufedjikh, Mahrouz, Amiot, & Lacroix, 2000) after *Citrus* fruits were harvested. After the post-harvest treatment of *Citrus* fruits with 1.5% oligochitosan, SOD, peroxidase (POD), CAT, ascorbate peroxidase (APX), polyphenoloxidase (PPO), and GR activities were increased (Huang, Ming, Deng, Deng, & Zhang, 2010). *Citrus* peels (CP) treated with methyl jasmonate and ethanol had higher levels of total phenolics, flavonoids, anthocyanins as well as higher radical scavenging activities against DPPH·, superoxide, and hydroxyl radicals than those in the control.

UV radiation, used in post-harvest as a sanitizing treatment can induce phenylpropanoid metabolism and biological stress in *Citrus* with the consequent production of phytoalexin compounds, such as flavonoids or stilbenes (Rodrigues, Pérez-Gregorio, García-Falcón, & Simal-Gándara, 2009).

4.5. Processing factors

Citrus fruits have been processed into various products, including cans, juices, wines, vinegars, jams and preserves. Methods of processing include heat-treatment and non-heat-treatment. The heat-treatment methods usually involved are blanching, sous vide, pasteurization, and canning. The non-heat-treatment processing methods include high-pressure processing, pulsed electric field, sonication, ozone and ultraviolet light.

There are several reports on the relationship between processing treatments and the antioxidant compounds or radical scavenging capacity in *Citrus* extracts and most studies have shown that whatever method of processing was used, the contents of phytochemical in *Citrus* fruits decreased. Keenan et al. (2010) found that both heat-treatment and non-heat-treatment caused a significant reduction of phenolic compounds, and higher temperatures lead to a decrease of vitamin C. The antioxidant activity of CP extracts was significantly affected by heating and processing time, therefore, the heating process can be used as a tool for increasing the antioxidant activity of CP. Sánchez-Moreno et al. (2005) found that freezing and pasteurization processes both led to diminished naringenin contents, while no modification in hesperetin levels. Del Caro et al. (2004) studied the minimally processed *Citrus* fruits of different species and cultivars ("Palazzelli" mandarin-type fruit, "Red blush" grapefruit, "Minneola" tangelo and "Salustiana" and "Shamouti" orange). They found that the antioxidant capacity of minimally processed segments and juices increased significantly in "Red blush" grapefruit juices and "Salustiana" orange segments, decreased in "Salustiana" juices and "Minneola" tangelo segments, and remained constant in the other samples.

To date, many studies have shown that high pressure and pulsed electric field technologies were more effective than high pressure treatment in preserving bioactive compounds in freshly squeezed orange juice (Keenan, Rößle, Gormley, Butler, & Brunton, 2012). Alkalinity of the solution system is the main factor affecting the rate of oxidation of polyphenols (Jeong et al., 2004). In addition, the levels of bioactive compounds in fresh juices made by hand are superior to those made by machines. Furthermore, packaging materials also affect the antioxidant activity of *Citrus* products, for instance, juice packaged in cardboard cartons contains a higher quantity of furocoumarins than that of cans and plastic cases (Girennavar et al., 2008).

5. Mechanism of antioxidant action of *Citrus* fruits

5.1. General

For the complex natural products and their diverse compounds, many different mechanisms of antioxidant action have been suggested in the existing literature (López-Alarcón & Denicola, 2013). To analyze the mechanisms of antioxidant action in *Citrus* fruits, which have rarely been explored, we brought together the literature information and summarized it in Fig. 1, which we based on our following discussion.

5.2. Inhibition of oxidant enzymes

Citrus fruits may exert their antioxidant capacity by inhibiting the oxidant enzymes via the bioactive compounds they contain (Fig. 1, pathway 1). Oxidant enzymes, such as nitric oxide synthase (NOS), lipoxygenase (LOX), xanthine oxidase (XO), cyclooxygenase (COX), NADPH oxidase (NOX), and myeloperoxidase (MPO), have played important roles in redox reactions of a biological system, and are also the main promoters of cellular ROS/RNS (López-Alarcón & Denicola, 2013). The inhibition of XO has been suggested to be one of the key mechanisms of antioxidant action in natural products (López-Alarcón & Denicola, 2013). In *Citrus*, Nakao et al. (2011) found that hesperetin can directly decrease cellular free radical production by inhibiting XO. Lin et al. (2008) reported that coumarins can directly decrease cellular free radical production by inhibiting XO. Therefore, polyphenols and other phytochemicals potentially contribute to the antioxidant capacity of *Citrus* fruits.

5.3. Interaction with redox signalling pathways

Citrus fruits may activate nuclear factor E2-related protein 2 (Nrf-2) transcription factor and inhibit nuclear factor kappa B (NF-κB) of the redox signalling pathway to exhibit their antioxidant activity (Fig. 1, pathway 2). The balance of cellular redox status is controlled by endogenous oxidants, such as H_2O_2, serving as second messengers and triggering intracellular cascade of signalling reactions, which spur the expression of detoxifying

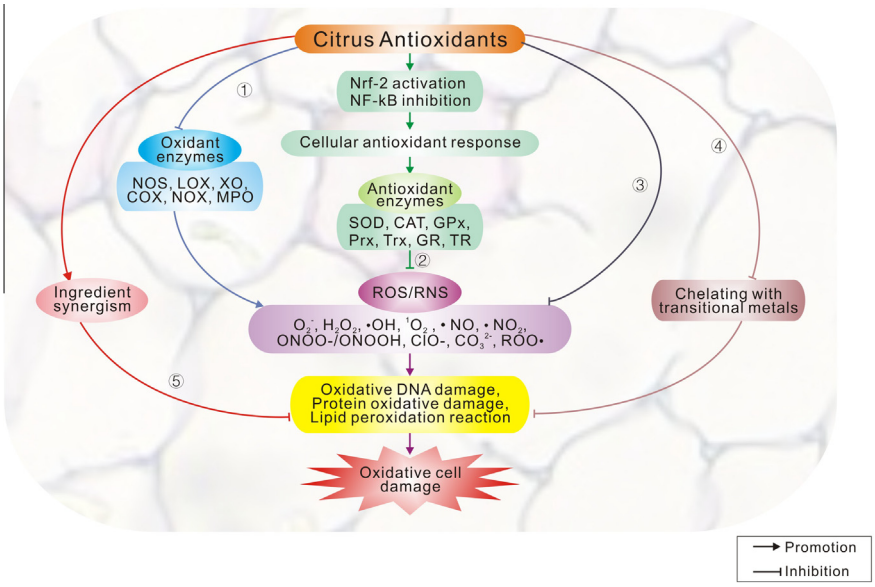


Fig. 1. A schematic representation of the potential antioxidant mechanism of action of *Citrus* fruits. Five pathways are suggested in existing literature: (1) inhibition of oxidant enzymes, reducing the cellular production of ROS/RNS, (2) interaction with redox signalling pathways, leading to the cellular antioxidant response, (3) direct reaction with ROS/RNS as a "free radical scavenger", (4) chelate with transitional metals yielding less oxidative damage, (5) interaction of the ingredient synergism, which influence the whole antioxidant system. Oxidant enzymes that produce ROS/RNS: NOS (nitric oxide synthase), LOX (lipoxygenase), XO (xanthine oxidase), COX (cyclooxygenase), NOX (NADPH oxidase), MPO (myeloperoxidase). Antioxidant enzymes: SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase), Prx (peroxiredoxin), Trx (thioredoxin), GR (glutathione reductase), TR (thioredoxinreductase).

enzymes and antioxidants (Forman, Maiorino, & Ursini, 2010). In the current literature, two key redox signalling pathways have been identified, i.e., Nrf-2 and NF-κB, which define the redox controls localized in the nucleus and cytosol, respectively (López-Alarcón & Denicola, 2013).

Nrf-2 is a redox-sensitive transcription factor and is activated by an oxidative signal in the cytoplasm, which causes translocation to the nucleus, where it binds to the antioxidant response elements DNA ARE-regions, and then induces the expression of cytoprotective enzymes like SOD, GSt, and NADPH-quinone oxidase (NQO) (HO-1) (Eggler, Gay, & Mesecar, 2008).

The NF-κB family consists of a group of inducible transcription factors which regulate immune and inflammatory responses, and prevent cells from undergoing apoptosis in response to oxidative stress. NF-κB is kept inactive by association with inhibitory IκB proteins in the cytoplasm. IκB proteins are rapidly degraded by the proteasome, liberating NF-κB proteins to the nucleus where they bind to specific DNA sequences, activating the expression of specific pro-inflammatory and anti-apoptotic genes in response to an inflammatory stimulus, including oxidants (Renard et al., 2000). Phytochemicals that decrease the expression of NF-κB and/or inhibit its activation prevent its translocation to the nucleus and the induction of pro-oxidant genes.

According to the literature, solated polyphenols like quercetin as well as extracts from natural products show an antioxidant response via activation of Nrf-2 (Arredondo et al., 2010; Tanigawa, Fujii, & Hou, 2007). In addition, inhibition of Nf-kB activation has been obtained by incubation of cell cultures to phenolic compounds from fruit extracts, such as blueberries (Xie et al., 2011).

Since *Citrus* fruits contain rich bioactive compounds, we suggest that the redox signalling pathway should be one of the most important ways by which *Citrus* fruits exhibit their antioxidant activity.

5.4. Direct reaction with ROS/RNS

Citrus bioactive compounds may directly react with ROS and/or reactive nitrogen species (RNS), and show the antioxidant activity of *Citrus* fruits (Fig. 1, pathway 3). ROS/RNS, which include mainly hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical ($^{\cdot}OH$), $^1O_2$, nitric oxide (NO), eroxynitrite ($ONOO^-$), hypochlorus acid (HOCl), carbonate (CO_3^{2-}) and peroxyl radical ($ROO^{\cdot}$), are involved in the growth, differentiation, progression and death of cells, and can react with proteins, enzymes, membrane lipids, nucleic acids, and other small molecules. A low amount of ROS/RNS is indispensable in intracellular signalling defence responses to pathogens, while, higher concentrations of ROS/RNS play a pivotal role in the pathogenesis of a lot of human diseases (Rajendran et al., 2014).

The phytochemicals in *Citrus* fruits may act as safeguard against the accumulation of ROS/RNS and eliminate them from the system. Through the irreversible dehydrogenation, vitamin C directly reacts with O_2^-, HOO^- and OH^-, clearing $^1O_2$ (Amitava & Kimberly, 2014). The antioxidant activity of vitamin E and carotenoids are also associated with the restraining of free radicals production and quenching $^1O_2$ (Amitava & Kimberly, 2014; Di Mascio et al., 1989; Levander et al., 1995). Flavonoids can act both as preventatives and chain scission factors at the same time, efficiently destroying the reactive oxygen species (Nakao et al., 2011). Moreover, limonoids work through reacting with free radicals to reduce their activity and prevent further chain reactions, and then to achieve the effect of free radical scavenging (Poulose et al., 2005). *Citrus* fruits rich in antioxidants should be good scavengers of ROS/RNS.

5.5. Chelators with transitional metals

Citrus fruits may chelate with transitional metals, and show antioxidant activities (Fig. 1, pathway 4). Lipid peroxidation is one of the major causes of oxidative cell damage. In oxidation, the action of metal ions is a main cause of lipid peroxidation reactions (Dávalos, Gómez-Cordovés, & Bartolomé, 2003). Of the metal ions, ferrous ions (Fe^{2+}) and copper ions (Cu^{2+}) are the most influential promoters that cause lipid peroxidation.

Vitamin E in *Citrus* fruits may chelate transitional metals, such as Fe^{2+}- and Cu^{2+} (Amitava & Kimberly, 2014). It was reported that

vitamin E from *Citrus* fruits reduced ferrous iron to ferric iron contents (Amitava & Kimberly, 2014).

5.6. Ingredient synergism

The interaction or synergistic effect among the nutrients and/or bioactive compounds contained in *Citrus* fruits may also contribute to their antioxidant capacity (Fig. 1, pathway 5). Besides the antioxidant capacity of natural phytochemicals themselves, the matrix in which they have to execute their function is also important (Arts et al., 2002). As we can see from this review, *Citrus* fruits are rich in various nutrients and bioactive compounds.

In a book chapter, the antioxidant synergism between vitamins themselves and with other phytochemicals were recorded in details (Amitava & Kimberly, 2014). It was reported that a combination of vitamin C and E can enhance antioxidant effects by means of vitamin C as a hydrogen donor. The synergistic effect of selenium and vitamin E protect mitochondria against free radical damage and their membranes against peroxidation damage. Under lipid soluble conditions, a small amount of vitamin E is enough to prevent the oxidation of carotenoid, enhancing their antioxidant capacity. Polyphenols had synergistic effects with vitamins C and E by their mutual reduction (Amitava & Kimberly, 2014). Besides these, the interaction between flavonoids and proteins influences the efficacy of the antioxidant was also reported (Arts et al., 2002). In the current literature, the interaction between naringenin and hesperidin of naval orange (*C. sinensis* Osbeck.) provides an example of the synergism among phenolic compounds (Freeman, Eggett, & Parker, 2010). Furthermore, the flavonols, quercetin and quercetin-3-glucoside trigger a noticeable increase in antioxidant activity when mixed in solution with another flavonoid (Hidalgo, Sánchez-Moreno, & Pascual-Teresa, 2010). Finally, we hope to point out that ingredient synergism should contribute to the total antioxidant capacity of *Citrus* fruits. However, to find the underlying mechanism of action, further work is obviously needed.

6. Concluding remarks

According to an old Chinese saying, herbs and foods are equivalent. In fact, *Citrus* plants have been used as traditional medicines for more than 1500 years in China. Although single administration of a high dose antioxidant may be harmful, the epidemiological studies have demonstrated that consumption of fruits and vegetables rich in antioxidants is beneficial to human health. From this review, we can see that *Citrus* fruits are a rich source of natural antioxidants.

Despite the huge amount of studies concerning the antioxidant activity of *Citrus* fruits and their role in human health that have been carried out in the past decades, some important scientific issues are still rarely addressed. For example, current studies are mainly focused on revealing the function of individual *Citrus* components and their antioxidant activities. The complexity of a plant matrix in which a single compound can function, and the synergism and antagonism make the study results hard to explain.

Along with the rapid development in purification and identification technologies of plant bioactive compounds, studies concerning *Citrus* bioactive compounds and antioxidant activities will attract more and more attention in the future. Currently, however, it is still hard to find a single, well applicable analysis method for *Citrus* fruits and their compounds. It is very important to standardize the current analytical methods and express the results as standard equivalents in order to make it possible to compare different studies from different laboratories. It is necessary to develop the simple, rapid and accurate *in vivo* methods for the antioxidant capacity evaluation of *Citrus* fruits.

For future study, the following fields should be paid more attention to break the bottlenecks. (1) The molecular and cellular mechanisms by which *Citrus* antioxidants function in a *in vivo* system should be clearly elucidated. (2) The synergism and/or antagonism between different antioxidants in *Citrus* fruits should be more explored. (3) The internal and external factors which influence the content of *Citrus* antioxidants and their activity during the fruits processing should be more studied. (4) New antioxidant compounds from *Citrus* fruits should be explored for a full use of rich *Citrus* germplasms.

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