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Dietary flavanones and citrus fruits influence cytokines and thyroid transcription factor-1 in an HDM-induced chronic asthma murine model

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ABSTRACT

The effects of hesperetin plus naringenin, orange juice and grapefruit juice on mRNA expression of TNF-α, TGF-β1 and TTF-1 in a house dust mite (HDM)-induced asthma animal model were investigated. Interventional mice exposed to HDM for 6 weeks received hesperetin plus naringenin, orange plus grapefruit juice, orange juice, grapefruit juice or water during the last 4 weeks, whereas non-asthmatic control mice consumed water. Lung tissue TNF-α and TGF-β1 expressions in supplemented groups were significantly reduced ($p < 0.0001$ and $p < 0.001$, respectively) compared with asthmatic controls. Hesperetin plus naringenin and orange plus grapefruit juice intake reduced expression of TGF-β1 more than grapefruit juice ($p \leq 0.02$) or orange juice compared with the asthmatic control group. All supplemented groups had non-significantly higher TTF-1 expression compared with water intake groups. Orange plus grapefruit juice or hesperetin plus naringenin with down-regulation of TNF-α and TGF-β1 may ameliorate lung damage in the lung tissue of asthmatic subjects.

1. Introduction

Chronic asthma is an epithelial disease characterized by inflammatory cells and structural changes in the airways throughout features of "remodelling" which is a result of persistent inflammation and epithelial damage in response to repetitive injury (Cho, 2011; Holgate et al., 2000). Many of the airway's structural elements alter to produce cytokines, growth factors, and mediators that may contribute to sustaining the inflammatory response (Holgate et al., 2000).

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TGF-β is one of the main mediators involved in tissue remodelling in the asthmatic lung. Exposure to reactive oxygen species has been shown to induce rapid cleavage of TGF-β1 from the latent complex. Although this process is essential for airway wound repair and healing, sustained activation of TGF-β1 inhibits beneficial TGF-β1 signalling and also activates fibroblasts and the epithelial–mesenchymal trophic unit, resulting in further release of reactive oxygen species and ultimately airway remodelling (Brown et al., 2012).

The TNF superfamily (TNFSF), including TNF-α, is another cytokine which might play a significant role in airway remodelling through the activation of NFκ-B, AP-1 and other transcription factors (Cazzola & Matera, 2011; Cho, 2011).

Thyroid transcription factor-1 (TTF-1), also known as Nkx2-1, is a homeodomain-containing transcription factor selectively expressed in the forebrain, thyroid, and lung (Boggaram, 2009). TTF-1 activates the expression of genes critical for lung development and function (Reynolds, Mucenski, & Whitsett, 2003). It has been shown to be suppressed in the airway epithelial cells of patients with asthma (Maeda et al., 2011). Recent studies exhibit that TTF-1 inhibits TGF-β–mediated epithelial–mesenchymal transition (EMT) and restores epithelial phenotype in lung adenocarcinoma cells. Conversely, enhancement of autocrine TGF-β signalling may accelerate the decrease of TTF-1 expression (Saito et al., 2009). The inhibitory effect of NKX2-1 was shown on mucous metaplasia and Th2 inflammation, the airway epithelial cells and in a mouse model (Maeda et al., 2011). TNF-α inhibits TTF-1 gene transcription and promoter activity, indicating that transcriptional mechanisms play important roles in the inhibition of the TTF-1 level (Das et al., 2011).

Multi-factorial processes have been implicated in the rising incidence of asthma. Changes in environmental factors such as diet are one of the several factors in this trend, which result in changes in gene–environment interactions (Devereux, 2010; Miller, 2001). Indeed, foods include both allergy-promoting and anti-allergic nutrients (Kawai et al., 2007). More than a thousand polyphenol structural molecules including flavonoids as bioactive compounds have been distinguished in many edible plants. In terms of heterocyclic type, flavonoids are divided into six subclasses including flavonols, flavanones, isoflavones, flavanonesl, anthocyanins, and flavanols. Considering their basic skeletal structure they reveal diverse functions (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). Flavanones, hesperetin and naringenin, as an exclusive subclass of flavonoids, mainly found in citrus fruits such as orange and grapefruit, have been recognized to exert antioxidant, anti-inflammatory and anti-allergic effects. Recent investigations have been increasingly focused on their biological actions on cellular–molecular mechanisms. Hesperetin has been shown to suppress the number of total inflammatory cells and Th2 cytokines in bronchoalveolar lavage fluid (BALF) in vivo in ovalbumin (OVA) sensitized animal models (Yang et al., 2011a). Naringenin attenuates ovalbumin-induced airway inflammation, serum total IgE, mRNA levels of chemokine ligands (CCL5, CCL11), and iNOS, and inhibits NF-κB DNA-binding activity (Shi et al., 2009).

Despite the great importance of citrus fruit juice as a widely consumed dietary item, there have been a limited number of studies that evaluated its impact on risk of chronic diseases such as CVD and Alzheimer's or against chromosome damage. To the best of our knowledge, there have been few studies, if any, on their comparative mechanism on chronic asthma. Herein, we were interested in investigating and comparing the effects of synthetic flavanones, hesperetin [3′,5,7-trihydroxy-4′-methoxyflavanone, 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-chromanone] and naringenin [±-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, 4′,5,7-trihydroxyflavanone], and their natural source, orange juice and grapefruit juice on TNF-α, TGF-β1 and TTF-1 in a simultaneously house dust mite (HDM)-induced chronic asthma mouse model.

2. Materials and methods

2.1. Experimental protocol

Detailed methodology was performed as previously described (Seyedrezazadeh et al., 2015). Briefly, Moro Blood orange (*Citrus sinensis* var. Moro) and grapefruit, white (*Citrus paradisi*), were obtained at maturity. Hesperetin (≥95%, C16H14O6, Cat. No. W431300) and naringenin (≥95%, C15H12O5, Cat. No. N5893) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Fruits were squeezed; the juice obtained was filtered and stored at −20 °C in aliquots of 230 cc dark glass bottles. Every 2 days frozen juice aliquots were thawed and spilled in the bottle of each supplemented group cage instead of tap water. Hesperetin and naringenin supplements were prepared daily, in which each 100 ml of the prepared fluid contained 7 mg of hesperetin and 9 mg of naringenin in water.

48 male BALB/C mice (mean weight: 27.92 ± 2.02) were randomly divided into the following six groups (8 animals per cage): (1) tap water for the non-asthmatic control (NC) and asthma control (AC) groups; (2) hesperetin plus naringenin for the hesperetin–naringenin (HN) group; (3) mixture of orange and grapefruit juice for the orange–grapefruit (OGJ) group; (4) orange juice for the orange (OJ) group; (5) and grapefruit juice for the grapefruit (GJ) group. They were fed a commercially available food pellet diet (normal diet) and water ad libitum. All the procedures in this study were performed in accordance with the guidelines for the Care and Use of Laboratory Animals as adopted by the Ethics Committee of the Faculty of Veterinary Medicine of University of Tabriz (140/9970, March, 2012/ Faculty of Veterinary Medicine of University of Tabriz).

Chronic asthma HDM-induced remodelling was performed according to the methods of Doherty et al. (2011). Mice were exposed to purified *Dermatophagoides pteronyssinus* HDM whole-body extract (Greer Laboratories, Lenoir, NC, USA) intranasal on days 0, 7 and 14 with 200, 100 and 100 μg HDM respectively, each absorbed to 40 μl PBS. Using the 50 μg in 40 μl PBS were then performed two times per week for four weeks to allow progressive airway remodelling. Control, normal control group received only 40 μl PBS intranasal, according to the above schedule.

2.2. Sample collection

24 h after the last challenge, the mice were sacrificed by exsanguination (i.p.). The chest cavity was exposed to allow for expansion, after which the lungs were isolated and the inferior

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right lobe was cut carefully into slices less than 0.5 cm thick as quickly, then submerged in the micro-tubes containing RNAlater RNA Stabilization Reagent (Qiagen, TX, USA). Tissues were stored at –20 °C according to manufacturer instructions until use.

2.3. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) and qRT-PCR

Removed frozen tissues from RNAlater stabilized the reagent to mortar. Total RNA was isolated by adding 1 ml TRIzol reagent (Life Technologies, Rockville, MD, USA) and disrupting with a pestle. Then RNA purification followed according to the manufacturer's protocol RNeasy Mini Kit (Cat. No. 74104, Qiagen, TX, USA). First-strand cDNA synthesis was performed using a QuantiTect Reverse Transcription Kit (Cat. No. 205311, Qiagen, TX, USA) under the conditions of 42 °C for 15 min and 95 °C for 3 min.

First, Reverse transcriptase–polymerase chain reactions (PCRs) for TGF-β1, TNF-α, TTF-1 cDNA, amplification were used. GAPDH was used to standardize gene expression. The PCR was performed in 12 μl Master Mix Red, 4 μl distilled water, 1 μl each primer, and 2 μl of cDNA. The following primer pair was used for TGF-β1 forward, 5-AGCCCGAAGCGGACTACTAT-3, and reverse, 5-TCCACATGTTGCTCCACACT-3; for TNF-α forward, 5-GGCAGGTCTACTTTGGAGTCATTGC-3, and reverse, 5-ACATTCGAGGCTCCAGTGAATTCGG-3; for TTF-1 forward, 5-GGCCGCGGCCATGCAGCAGCAC-3, and reverse, 5-CCATG TTCTTGCTCACGTCC-3; and for GAPDH forward, 5-TTGTCAA GCTCATTTCCTGGTATG-3, and reverse, 5-GGATAGGGCCT CTCTTGCTCA-3. The profiles employed for PCR amplification comprised GAPDH: 94 °C for 5 min, 35 cycles of 94 °C for 40 s, 59 °C for 40 s, 72 °C for 40 s and a final extension cycle of 72 °C for 5 min; TGF-β1: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min; TNF-α and TTF-1: 95 °C for 60 s, 40 cycles of 95 °C for 30 s, 58 °C for 60 s, 72 °C for 60 s and 72 °C for 10 min.

Following completion of PCR, to validate the RT-PCR results, a quantitative real-time RT-PCR (qPCR) analysis was performed using Bio-Rad iQ5, Multicolor Real Time PCR Detection System and SYBR® Green. The qPCR was carried out using 10 μl Power SYBR® Green PCR Master Mix (2×), 8.4 μl distilled water, 0.6 μl of each primer and 1 μl cDNA, in triplicate reaction. The oligonucleotide primer sequences are shown in Table 1. The gene levels were normalized with GAPDH using the 2–ΔΔCT methods to calculate relative changes.

2.4. Statistical analysis

Data were analysed using IBM SPSS, Version 20.0 (SPSS, Inc., Chicago, IL, USA). Comparisons between groups were done using ANOVA and Tukey tests. All the data in the figures were expressed as mean ± SD from triplicate assays. Differences were considered to be statistically significant when $p < 0.05$.

3. Results

The expression of TGF-β1, TNF-α and TTF-1 in lung tissue was determined after the intervention of naringenin–hesperetin, or orange and grapefruit juices in the mouse model of HDM-induced chronic asthma.

A non-significant decreased expression of TNF-α was observed in supplemented groups compared with the non-asthmatic control (NC) group (Fig. 1A). But TNF-α expression in lung tissue in supplemented groups was significantly reduced compared with the asthmatic control (AC) group ($p < 0.0001$). No significant fold change differences were shown between intervention groups in the TNF-α expression compared with the non-asthmatic control (NC) group (Fig. 1C). However, down-regulation of TNF-α expression in HN and OGJ groups was three and five fold changes more than the GJ group compared with the asthmatic control (AC) group, respectively (Fig. 1D).

Oral administration of HN, OGJ, OJ and GJ significantly reduced TGF-β1 expression compared with NC ($p \leq 0.02$) (Fig. 2A) and AC groups ($p < 0.001$) (Fig. 2B). HN and similarly OGJ resulted in down-regulation of TGF-β1 of twenty and two-fold more than GJ ($p \leq 0.02$) and OJ compared with non-asthmatic control (NC) and asthmatic control (AC) groups, respectively (Fig. 2C and D).

We further investigated the effect of HDM-chronic asthma on TTF-1 expression; however, there were no significant changes in TTF-1 expression in the supplemented groups compared with the NC and AC groups despite the higher expression of TTF-1 in the GJ group (Fig. 3).

There were no haematologic and total protein differences between groups (Fig. 4). However, the absolute number of eosinophil was significantly higher in the OGJ group than in NC, AC, HN, and GJ groups ($p < 0.02$) (Fig. 4).

In histopathological evaluation, consistent with previous studies (Seyedrezazadeh et al., 2015), the consumption of hesperetin–naringenin was associated with substantial amelioration of airway structural changes and suppressed inflammation. These changes were more significant in the case of hesperetin–naringenin consumption compared with fruit juice (Fig. 5).

4. Discussion

The major finding of the present study was that determined intervention of naringenin–hesperetin, or orange and grapefruit juices, significantly down-regulated the expression of TNF-α and TGF-β1. The reduction of TNF-α was non-significant between supplemented groups, but was more noticeable in the OGJ and OJ groups than in the HN group. However, administration of HN and OGJ significantly reduced the expression of TGF-β1 in comparison with GJ and OJ intake. Furthermore, our findings did not show a significant difference in lung TTF-1 expression between the intervention groups and the asthmatic control group; however, higher TTF-1 up-regulation was observed in GJ group than OJ and HN groups.

Flavanones exhibit biological properties such as anti-inflammatory and anti-fibrotic activities through inhibition or regulation of the signalling pathway. Recent studies have shown a significant dose-dependent decrease in the level of cytokines IL-2, IL-4, IL-5 and TNF-α by hesperetin intake in an OVA-induced animal model (Yang et al., 2011a). Naringenin disrupts TGF-β1 signalling by selectively targeting Smad3 in liver fibrosis (Liu et al., 2006).

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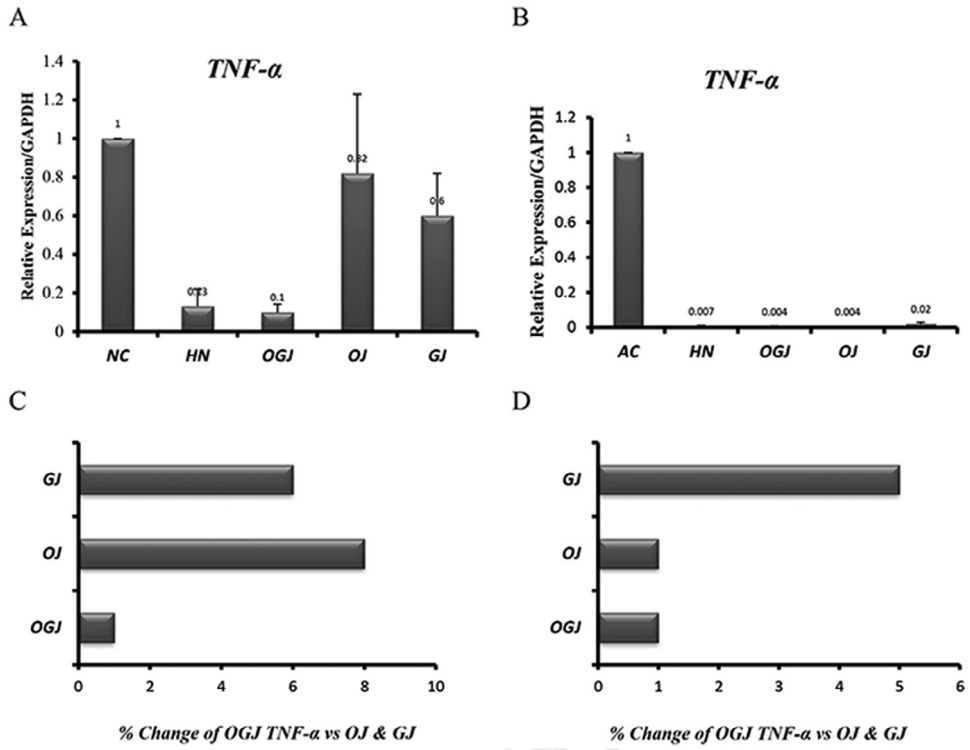

Fig. 1 – Effects of chronic HDM-induced asthma on TNF-α expression. (A) Relative expression value of supplemented groups compared with the non-asthmatic control group; (B) relative expression value of supplemented groups compared with the asthmatic control group; (C) fold-change values compared with the non-asthmatic control group; (D) fold-change values compared with the asthmatic control group. Data represented as means ± SDs or %. Analysis was performed by one-way ANOVA.* $p < 0.0001$. NC, non-challenged control group; AC, asthma control group; HN, mixture of hesperetin and naringenin group; OGJ, mixture of orange and grapefruit juice group; OJ, orange juice group; GJ, grapefruit juice group.

According to our knowledge, none of the previous studies have evaluated the effects of citrus fruits in the HDM-induced chronic asthma animal model. However, some studies have shown the favourable impact of orange juice and grapefruits on chronic disease. Daily intake of 500 ml orange juice for two weeks in non-diabetic patients who were susceptible to cardiovascular disease could significantly decrease the plasma level of TNF-α and IL-6 (Buscemi et al., 2012). Zanotti et al. conducted an ex vivo study to investigate the effect of orange juice (OJ) and hesperidin (Hsd) on LPS induced macrophages. OJ decreased the level of TNF-α and NO, but no changes in IL-10 and IL-12 were observed on LPS induced macrophages. In this study, also, Hsd suppresses the LPS induced inflammatory response while OJ increases the anti-microbial activity of macrophages (Zanotti Simoes Dourado, de Abreu Ribeiro, Zeppone Carlos, & Borges Cesar, 2013).

Citrus fruits contain bioactive components such as high amounts of flavonoids, including polymethoxylated flavones and flavanones, and different levels of limonoids, carotenoids, ascorbic acid and potassium (Protti, Valle, Poli, Raggi, & Mercolini, 2015). In addition, these fruits have been known as rich sources of vitamin C, folate and β-carotene. Also, another type of flavonoid, anthocyanin, exists in red orange. It is evident that variation of bioactive components of citrus fruit depends on growing area, soil conditions etc. A study analysed the flavonoid content of non-concentrate orange and grapefruit juices in the US markets (Vanamala, Reddivari, Yoo, Pike, & Patil, 2006). It was found that hesperidin was the major flavanone, followed by narirutin and didymin, in orange juice. Also, in all brands of grapefruit juices, naringin, narirutin, and poncirin were the major flavonoids. The current study used the amount of hesperetin and naringenin supplementation according to USDA database for the flavonoid content of food (Department of Agriculture, January 2007). Vitamin C effect on significant reduction of TNF-α has been reported in pneumonia patients and in NF-κB LPS-stimulated macrophage cells (Chen et al., 2014). Reduced expression of TGF-β by vitamin C, folate and anthocyanin has been reported (Cao et al., 2013; Dundar et al., 2014; Li et al., 2012), but the therapeutic effectiveness of β-carotene is not clear. In recent studies, the increased level of intracellular TGF-β1 was attributed to the effects of β-carotene (Comerci et al., 1997).

There is some evidence that supports the role of TTF-1 as a critical factor in alveolar cell growth and development and also in alveolar structure repair after lung diseases or lung injury (Takahashi et al., 2010). Maeda et al. showed the suppressed level of TTF-1 in airway epithelial cells from asthmatic patients. Further morphometric analysis of OVA sensitized NKX2-1+/– mice, demonstrated increased mucous cell metaplasia compared with control mice. In contrast, the reduced level of NKX2-1 enhanced mucous metaplasia in airways. A study by Maeda et al. demonstrated that NKX2-1 inhibits goblet cell metaplasia and mucus secretion by inhibiting SPDEF and MUC5AC expression (Maeda et al., 2011). Histopathological

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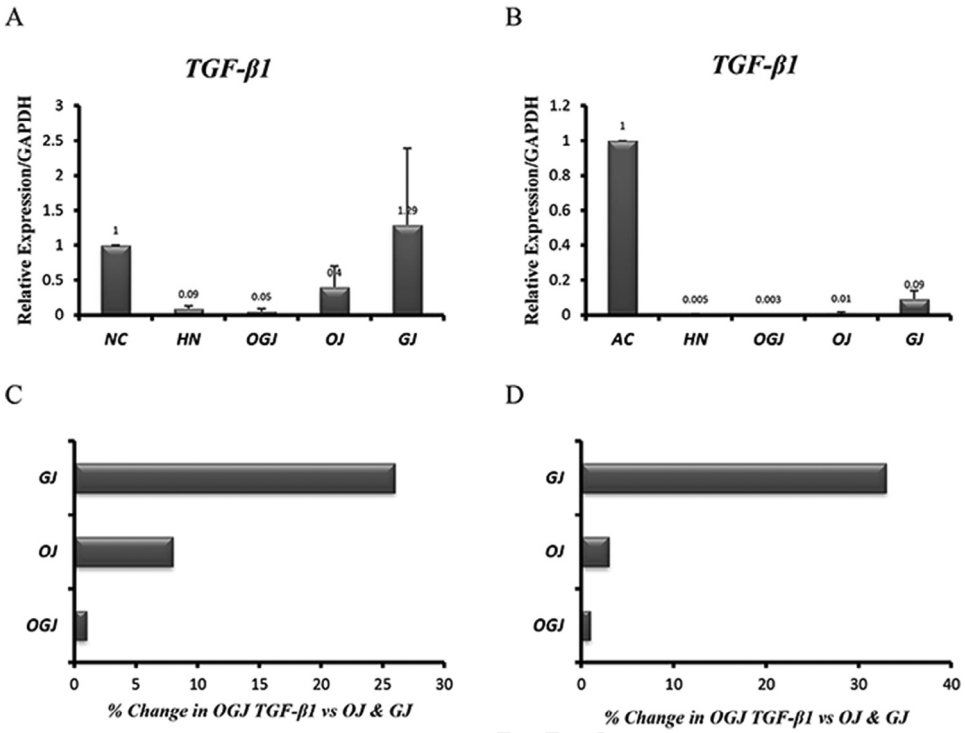

Fig. 2 – Effects of chronic HDM-induced asthma on TGF-β1 expression. (A) Relative expression value of supplemented groups compared with the non-asthmatic control group; (B) relative expression value of supplemented groups compared with the asthmatic control group; (C) fold-change values compared with the non-asthmatic control group; (D) fold-change values compared with the asthmatic control group. Data represented as means ± SDs or %. Analysis was performed by one-way ANOVA. NC, non-challenged control group; AC, asthma control group; HN, mixture of hesperetin and naringenin group; OGJ, mixture of orange and grapefruit juice group; OJ, orange juice group; GJ, grapefruit juice group.

findings of this study revealed the goblet cell metaplasia in the control asthma group, but not in the intervention groups despite the presence of mucous plugs. Therefore, increased expression of TTF-1 is likely to inhibit the goblet cell metaplasia.

This study highlights the possible effects of those genes involved in inflammatory responses and airway remodelling on histopathology changes. The effects of intervention supplements on TTF-1, TGF-β1, and TNF-α are discussed in more detail based on their bioavailability and synergic effects (Fig. 6).

Overproduction of ROS by alveolar macrophages, eosinophil, and inflammatory and epithelial cells results in hyperresponsiveness (AHR), microvascular hyperpermeability, lung injury and remodelling in asthma. The lung has a well-developed enzymatic antioxidant system such as GPx and SOD, and a non-enzymatic antioxidant system such as vitamin C and E that act as radical scavengers. The insufficiency of antioxidant function and the high level of reactive species (ROS/RNS) result in disturbance of the OX/Anti-OX balance in asthma (Mikolka, Mokra, Drgova, Petras, & Mokry, 2012). Therefore, reactive species may directly damage proteins, lipids and DNA, and can activate members of mitogen-activated protein kinase (MAPK) signalling such as Erk, P38 MAPK, and PI3K (Comhair & Erzurum, 2010), and NF-κB promotes inflammation (Uchida et al., 1999).

Flavonoids serve multiple roles in a variety of biological processes such as free radical scavenging, removal of metal ions, regulation of gene expression and signalling pathways, and suppression of inflammatory responses. Their role in the regulation of mitochondrial function has also been described (Lago et al., 2014). The chemical structure, concentration, and metabolites of flavonoids influence their antioxidant efficiency and radical scavenging capacity (Lago et al., 2014). H atoms in these phenolic groups are easily accessible. Therefore they can delocalize consecutive radicals on the flavonoid skeleton. The chemical nature of flavonoids relies on so many criteria including polymerization degree, hydroxylation degree, structural class, and other substitutions and conjugations. The antioxidant activity depends more on structural arrangement of substituents, configuration and number of hydroxyl groups, rather than on flavones' original structure. This fact is more prominent in flavonones with a substitution of the neohesperidose (ramnosil a-1, 2 glucose) group in the 7th position. But then again, the power of antioxidant was clearly raised by the 3′,4′-catechol structure, in the flavanones glycosylated with a neohesperidose of the 7th OH group. It has been shown in studies that the antioxidant activity of hesperetin is higher than glycoside form, but it dismiss when comprised between naringenin and its glycoside form. It could be assumed that radical-scavenging activities get influenced by O-glycosylation at hydroxyl position. On the other hand the sugar molecule located in 7th position is capable to correlate with the methoxyl group in the 4th position and modify the antioxidant power (Majo et al., 2005). Moreover, strong interaction has been suggested between endogenous and exogenous

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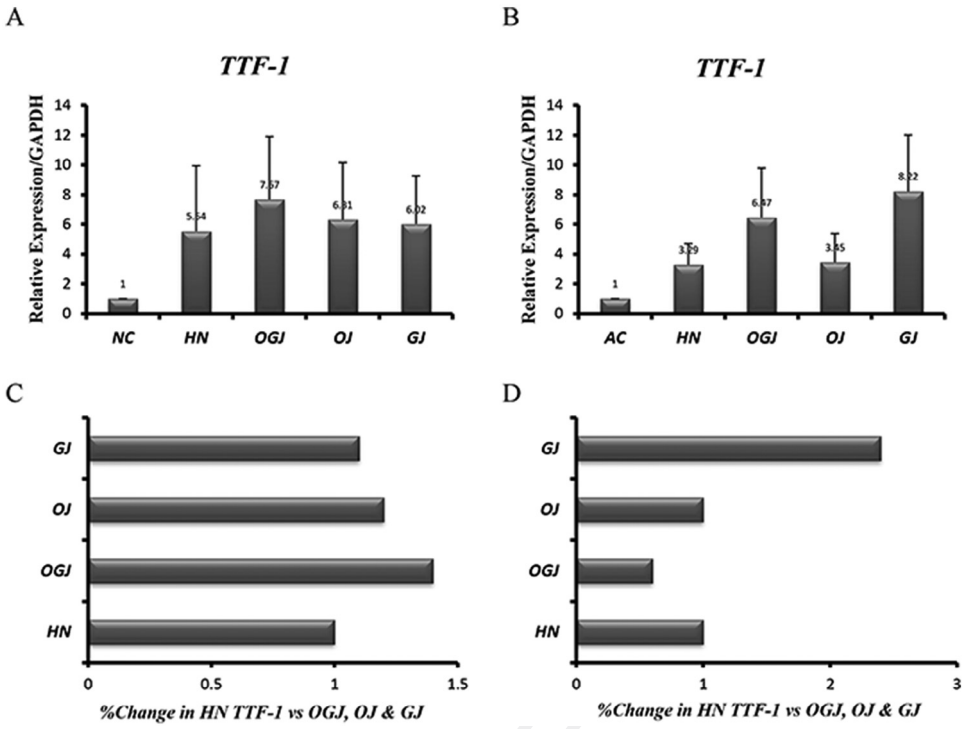

Fig. 3 – Effects of chronic HDM-induced asthma on TTF-1 expression. (A) Relative expression value of supplemented groups compared with the non-asthmatic control group; (B) relative expression value of supplemented groups compared with the asthmatic control group; (C) fold-change values compared with the non-asthmatic control group; (D) fold-change values compared with the asthmatic control group. Data represented as means ± SDs or %. Analysis was performed by one-way ANOVA. NC, non-challenged control group; AC, asthma control group; HN, mixture of hesperetin and naringenin group; OGJ, mixture of orange and grapefruit juice group; OJ, orange juice group; GJ, grapefruit juice group.

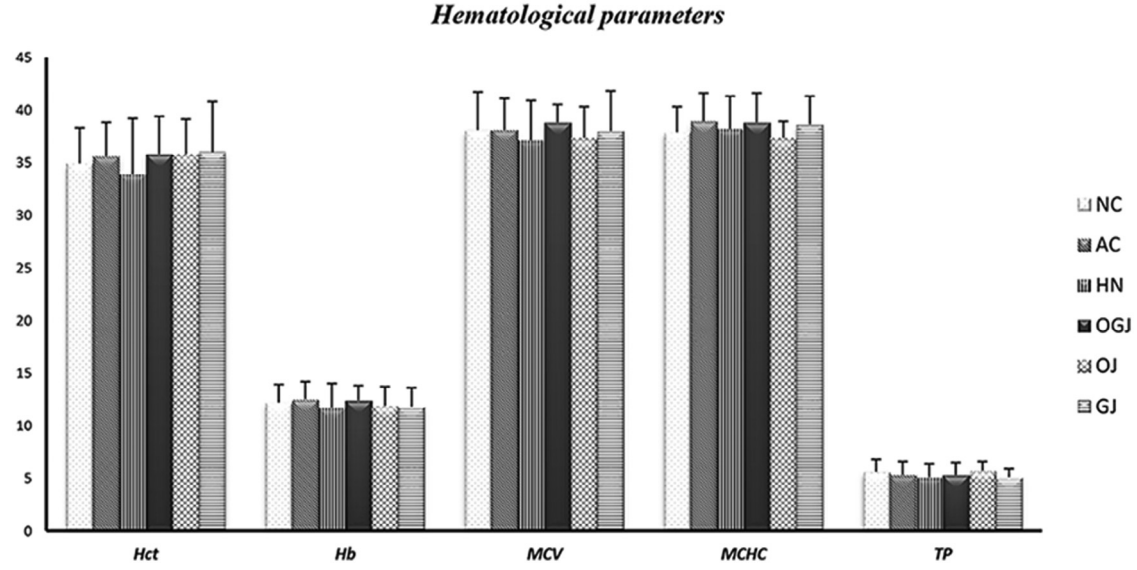

Fig. 4 – Haematologic parameters and cell blood count in chronic HDM-induced asthma male mice. Data represented as means ± SDs. Analysis was performed by one-way ANOVA. NC, non-challenged control group; AC, asthma control group; HN, mixture of hesperetin and naringenin group; OGJ, mixture of orange and grapefruit juice group; OJ, orange juice group; GJ, grapefruit juice group.

antioxidants. This coordination is achieved, at least in part, through antioxidant responsive elements (AREs), which are found in the promoters of many of the genes (Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005). Therefore, pharmacologic activity of flavonoids modulates several cell functions. Citrus fruits, as flavonoid sources, are considered to have antioxidant and anti-mutagenic activities and to have beneficial effects on cardiovascular, skeletal, and immune systems

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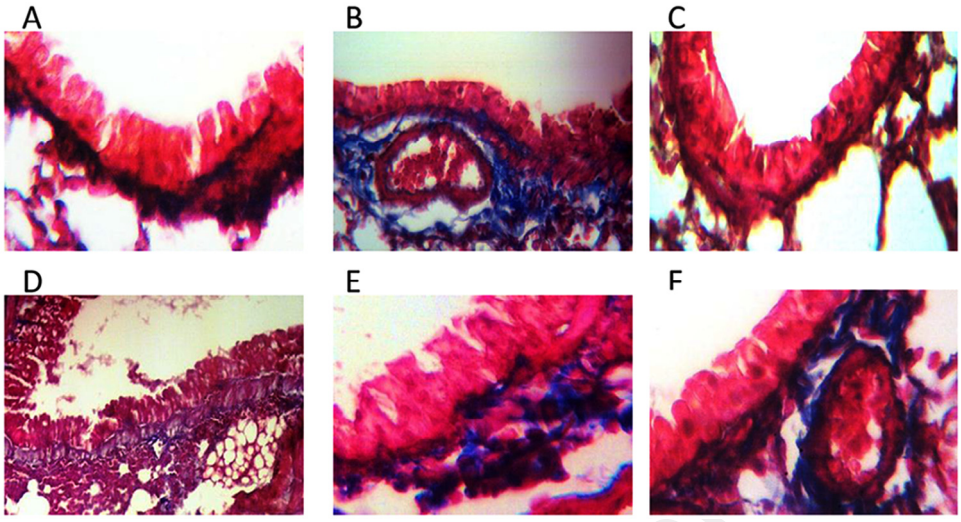

Fig. 5 – Effects of grape and orange juice and hesperetin–naringenin supplementation on airway remodelling in chronic HDM-induced asthma male mice. Representative histopathological examination Masson's trichrome (MT) staining of lung section from the (A) non-challenged control (NC) group; (B) asthmatic control (AC) group; (C) mixture of hesperetin and naringenin (HN) group; (D) mixture of orange and grapefruit juice (OGJ) group; (E) orange juice (OJ) group; and (F) grapefruit juice (GJ) group.

(Perche et al., 2014). Findings have shown significant effects of hesperetin–naringenin and orange juice–grapefruit juice mixtures on the down-regulation of TNF-α and TGF-β1. In addition the suppressing effect of orange juice and grapefruit juice on tissue and airway inflammation and less effectiveness of orange and grapefruit juice mixture on the decrease of eosinophil indicated that hesperetin–naringenin is a more efficient antioxidant and free radical scavenger than orange juice and grapefruit juice. On the other hand, the synergic effect of multiple medicines may increase their pharmacologic impact. Orange–grapefruit juice mixture or hesperetin–naringenin is likely to be synergistic in the regulation of TGF-β1and TNF-α as we observed higher down-regulation of these genes in OGJ and HN groups.

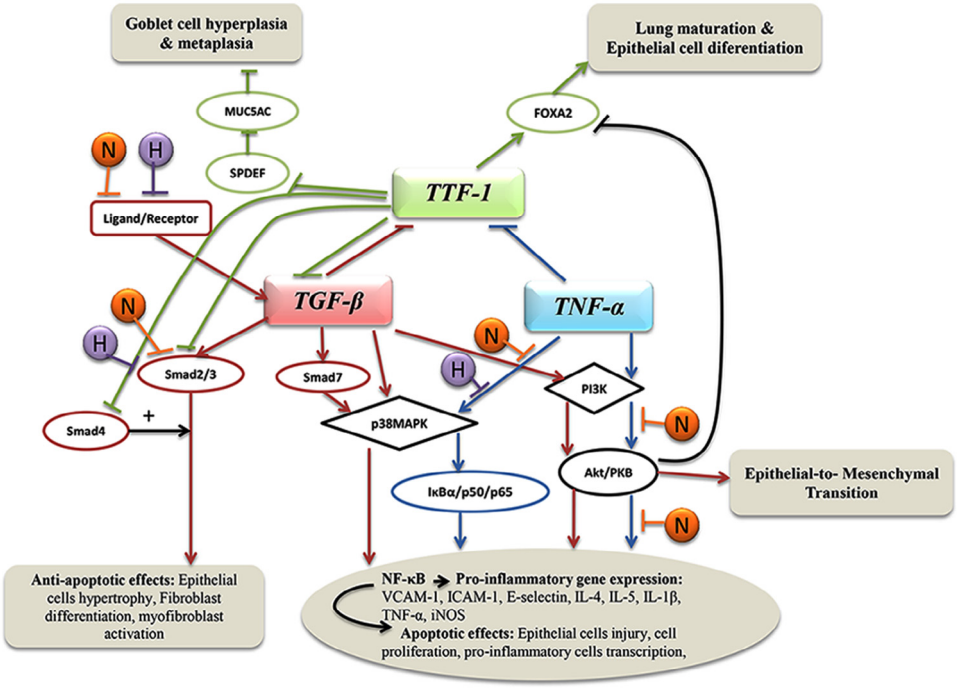

Fig. 6 – Possible interaction signalling way between TTF-1, TNF-α and TGF-β1; and possible effectiveness of consumption of hesperetin and naringenin.
Naringenin Hesperetin

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TGF-β1 and TNF-α are key mediators in airway remodelling and inflammation. The primary pathway of TGF-β1 is the Smad-protein mediated pathway. However, other intracellular proteins including MAPKs, depending on cell type and microenvironmental conditions, activate TGF-β1 (Makinde, Murphy, & Agrawal, 2007). Environmental risk factors trigger pro-inflammatory mediators such as TNF-α and activate intracellular MAPK/NF-κB and related signal pathways including protein kinase C and PI3K. The activation of these pathways stimulates the proliferation of airway cells and production of cytokines which are involved in airway remodelling, gene expression and mucin secretion (Zhang, Cardell, Edvinsson, & Xu, 2013). A large number of studies have shown the biological effects of flavanones on inflammatory cytokines (Moos & Kim, 2012). Hesperetin reduces the level of proinflammatory mediators TNF-α, IL-1α, IL-1β, and IL-6 in LPS-stimulated human PBMCs (peripheral blood mononuclear cells) (Fordham, Naqvi, & Nares, 2014; Li et al., 2013).

Naringenin has direct and indirect antifibrogenic effects which are mediated by down-regulation of Smad3 protein and phosphorylation through TGF-β1 signalling (Liu et al., 2006). Naringenin decreases ECM formation induced by TGF-β1, including collagen-Iα1 (Col Iα1), fibronectin, and PAI-1 (plasminogen activator inhibitor-1). In addition, it reduces the mRNA and protein expression of Smad3 induced by TGF-β1 and down-regulates EMT markers by inhibiting the TGF-β1/Smad3 signal pathway (Liu et al., 2006; Lou et al., 2012). Moreover, naringenin inhibits Akt that is the inhibition of LPS-induced NF-κB via inactivation of PI3K/Akt signalling pathway (Park, Kim, & Choi, 2012). Using single-molecule fluorescence imaging and single-molecule force measurement, Yang et al. found that the natural compound naringenin or hesperetin inhibits TGF-β1 ligand–receptor interaction which, in turn, inhibits the receptor dimerization, signalling complex formation, and subsequent Smad3 phosphorylation for the downstream signal transduction (Yang, Wolfram, Shen, Fang, & Ferrari, 2012; Yang et al., 2011b).

Chronic exposure of alveolar epithelial cells to the combination of TGF-β1 and TNF-α results in decreased TTF-1 immunoreactivities with a resultant increase in α-SMA and other mesenchymal markers, indicating the induction of an EMT which could be responsible for the accumulation of fibroblasts and myofibroblasts (Isogaya et al., 2014). A study by Saito et al. showed that TTF-1 inhibits TGF-β mediated EMT and restores epithelial injury in lung adenocarcinoma. These findings describe potential links between TTF-1 and TGF-β signalling in lung disease through regulation of EMT and mesenchymal–epithelial transition (MET) (Saito et al., 2009). Isogaya et al. (2014) revealed that TTF-1 co-localizes with Smad3 on chromatin and alters Smad3-binding patterns throughout the genome, while TTF-1 generally inhibits Smad4 binding to chromatin. They suggest TTF-1 may compete with Smad4 for interaction with Smad3, and in the presence of TTF-1, Smad3 regulates the transcription of certain genes independently of Smad4 (Isogaya et al., 2014). Also, TTF-1 inhibits aeroallergen-induced mucous cell metaplasia by inhibiting SPDEF and MUC5AC and maintaining the expression of FOXA2 (Maeda et al., 2011). A comparison between the findings of this study and those of previous studies regarding the role of TTF-1 in TGF-β and TNF-α signalling pathways and also the possible effect of hesperetin–naringenin on these pathways is shown in Fig. 6.

Although this study was done for the first time with concurrent of citrus fruits and their flavanones on chronic asthma, it has some potential limitations. Non-measuring the protein of TNF-α, TGF-β1, and TTF-1. Simultaneous with their mRNA expression, assessment of AHR and Th2 cytokine production from CD4 T-cells are considered as limitations of this study. Also it seems that further long-time exposure to HDM than this method provides further valuable and detailed information on diet's role in airway remodelling and pathological markers in chronic asthma.

5. Conclusion

Findings of this study showed that OGJ and HN intake resulted in the down-regulation of pre-inflammatory cytokines including TGF-β1 and TNF-α and the up-regulation of TTF-1. Since TGF-β1 and TNF-α play a crucial role in airway remodelling and TTF-1 in alveolar structure repair, which in turn result in improving lung tissue growth, development, and repair. It is assumed the simultaneous intake of two different fruit juices or two flavanones examined in this study may produce a greater effect in the long term because of their synergic pharmacologic activities.

Conflict of interest

The authors have no relevant conflict of interests to declare.

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The authors' contributions are as follows: E. S., E. S., M. V., M. N, K. A. and M. P. M. developed the conception and methodology of this work; S. K., A. A. S. and E. S. designed and performed the animal experiments; S. G., A. S., A. B and E. S. contributed the reagents, materials and tools for the analysis; E. S. and M. A. J. performed the statistical analysis; and E.S., A. S. and M. P. M wrote the paper. Due to journal criteria for the number of authors, we would like to thank here Saied Ghorbian, Mohammad Najafi, Mohammad Asghari Jafarabadi and Masoud Pour Moghaddam for valuable contribution.

REFERENCES

Boggaram, V. (2009). Thyroid transcription factor-1 (TTF-1/Nkx2.1/TITF1) gene regulation in the lung. *Clinical Science*, *116*(1), 27–35.

Brown, S. D., Baxter, K. M., Stephenson, S. T., Esper, A. M., Brown, L. A., & Fitzpatrick, A. M. (2012). Airway TGF-beta1 and

oxidant stress in children with severe asthma: Association with airflow limitation. *The Journal of Allergy and Clinical Immunology*, *129*(2), 388–396, 396 e381-388.

Cao, L., Lou, X., Zou, Z., Mou, N., Wu, W., Huang, X., & Tan, H. (2013). Folic acid attenuates hyperhomocysteinemia-induced glomerular damage in rats. *Microvascular Research*, *89*, 146–152.

Cazzola, M., & Matera, M. G. (2011). Inhibiting or blocking LIGHT, a TNF superfamily member, for treating airway remodeling. *Expert Review of Respiratory Medicine*, *5*(5), 623–625.

Chen, Y., Luo, G., Yuan, J., Wang, Y., Yang, X., Wang, X., Li, G., Liu, Z., & Zhong, N. (2014). Vitamin C mitigates oxidative stress and tumor necrosis factor-alpha in severe community-acquired pneumonia and LPS-induced macrophages. *Mediators of Inflammation*, 2014, 426740.

Cho, J. Y. (2011). Recent advances in mechanisms and treatments of airway remodeling in asthma: A message from the bench side to the clinic. *The Korean Journal of Internal Medicine*, *26*(4), 367–383.

Comerci, J. T., Jr., Runowicz, C. D., Fields, A. L., Romney, S. L., Palan, P. R., Kadish, A. S., & Goldberg, G. L. (1997). Induction of transforming growth factor beta-1 in cervical intraepithelial neoplasia in vivo after treatment with beta-carotene. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *3*(2), 157–160.

Comhair, S. A., & Erzurum, S. C. (2010). Redox control of asthma: Molecular mechanisms and therapeutic opportunities. *Antioxidants & Redox Signaling*, *12*(1), 93–124.

Das, A., Acharya, S., Gottipati, K. R., McKnight, J. B., Chandru, H., Alcorn, J. L., & Boggaram, V. (2011). Thyroid transcription factor-1 (TTF-1) gene: Identification of ZBP-89, Sp1, and TTF-1 sites in the promoter and regulation by TNF-alpha in lung epithelial cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *301*(4), L427–L440.

Department of Agriculture, U.S. USDA Database for the Flavonoid Content of Selected Foods, Release 2.1. (January 2007). <http://www.ars.usda.gov/nutrientdata>.

Devereux, G. (2010). Session 1: Allergic disease: Nutrition as a potential determinant of asthma. *The Proceedings of the Nutrition Society*, *69*(1), 1–10.

Doherty, T. A., Soroosh, P., Khorram, N., Fukuyama, S., Rosenthal, P., Cho, J. Y., Norris, P. S., Choi, H., Scheu, S., Pfeffer, K., Zuraw, B. L., Ware, C. F., Broide, D. H., & Croft, M. (2011). The tumor necrosis factor family member LIGHT is a target for asthmatic airway remodeling. *Nature Medicine*, *17*(5), 596–603.

Dundar, R., Inan, S., Muluk, N. B., Cingi, C., Ilknur, A. E., & Katilmis, H. (2014). Inhibitory effect of N-acetyl cysteine and ascorbic acid on the development of myringosclerosis: An experimental study. *International Journal of Pediatric Otorhinolaryngology*, *78*(7), 1019–1025.

Fordham, J. B., Naqvi, A. R., & Nares, S. (2014). Leukocyte production of inflammatory mediators is inhibited by the antioxidants phloretin, silymarin, hesperetin, and resveratrol. *Mediators of Inflammation*, 2014, 938712.

Holgate, S. T., Davies, D. E., Lackie, P. M., Wilson, S. J., Puddicombe, S. M., & Lordan, J. L. (2000). Epithelial-mesenchymal interactions in the pathogenesis of asthma. *The Journal of Allergy and Clinical Immunology*, *105*(2 Pt. 1), 193–204.

Isogaya, K., Koinuma, D., Tsutsumi, S., Saito, R. A., Miyazawa, K., Aburatani, H., & Miyazono, K. (2014). A Smad3 and TTF-1/NKX2-1 complex regulates Smad4-independent gene expression. *Cell Research*, *24*(8), 994–1008.

Kawai, M., Hirano, T., Higa, S., Arimitsu, J., Maruta, M., Kuwahara, Y., Ohkawara, T., Hagihara, K., Yamadori, T., Shima, Y., Ogata, A., Kawase, I., & Tanaka, T. (2007). Flavonoids and related compounds as anti-allergic substances. *Allergology International*, *56*(2), 113–123.

Lago, J. H., Toledo-Arruda, A. C., Mernak, M., Barrosa, K. H., Martins, M. A., Tiberio, I. F., & Prado, C. M. (2014). Structure-activity association of flavonoids in lung diseases. *Molecules: A Journal of Synthetic Chemistry and Natural Product Chemistry. [electronic resource]*, *19*(3), 3570–3595.

Li, J., Kang, M. K., Kim, J. K., Kim, J. L., Kang, S. W., Lim, S. S., & Kang, Y. H. (2012). Purple corn anthocyanins retard diabetes-associated glomerulosclerosis in mesangial cells and db/db mice. *European Journal of Nutrition*, *51*(8), 961–973.

Li, R., Cai, L., Xie, X. F., Peng, L., Wu, T. N., & Li, J. (2013). 7,3′-Dimethoxy hesperetin inhibits inflammation by inducing synovial apoptosis in rats with adjuvant-induced arthritis. *Immunopharmacology and Immunotoxicology*, *35*(1), 139–146.

Liu, X., Wang, W., Hu, H., Tang, N., Zhang, C., Liang, W., & Wang, M. (2006). Smad3 specific inhibitor, naringenin, decreases the expression of extracellular matrix induced by TGF-beta1 in cultured rat hepatic stellate cells. *Pharmaceutical Research*, *23*(1), 82–89.

Lou, C., Zhang, F., Yang, M., Zhao, J., Zeng, W., Fang, X., Zhang, Y., Zhang, C., & Liang, W. (2012). Naringenin decreases invasiveness and metastasis by inhibiting TGF-beta-induced epithelial to mesenchymal transition in pancreatic cancer cells. *PLoS ONE*, *7*(12), e50956.

Maeda, Y., Chen, G., Xu, Y., Haitchi, H. M., Du, L., Keiser, A. R., Howarth, P. H., Davies, D. E., Holgate, S. T., & Whitsett, J. A. (2011). Airway epithelial transcription factor NK2 homeobox 1 inhibits mucous cell metaplasia and Th2 inflammation. *American Journal of Respiratory and Critical Care Medicine*, *184*(4), 421–429.

Majo, D. D., Giammanco, M., Guardia, M. L., Tripoli, E., Giammanco, S., & Finotti, E. (2005). Flavanones in citrus fruit: Structure–antioxidant activity relationships. *Food Research International*, *38*(10), 1161–1166.

Makinde, T., Murphy, R. F., & Agrawal, D. K. (2007). The regulatory role of TGF-beta in airway remodeling in asthma. *Immunology and Cell Biology*, *85*(5), 348–356.

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: Food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*(5), 727–747.

Masella, R., Di Benedetto, R., Vari, R., Filesi, C., & Giovannini, C. (2005). Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathione-related enzymes. *The Journal of Nutritional Biochemistry*, *16*(10), 577–586.

Mikolka, P., Mokra, D., Drgova, A., Petras, M., & Mokry, J. (2012). Dimethyl sulfoxide in a 10% concentration has no effect on oxidation stress induced by ovalbumin-sensitization in a guinea-pig model of allergic asthma. *Journal of Physiology and Pharmacology*, *63*(2), 179–186.

Miller, A. L. (2001). The etiologies, pathophysiology, and alternative/complementary treatment of asthma. *Alternative Medicine Review: A Journal of Clinical Therapeutic*, *6*(1), 20–47.

Park, H. Y., Kim, G. Y., & Choi, Y. H. (2012). Naringenin attenuates the release of pro-inflammatory mediators from lipopolysaccharide-stimulated BV2 microglia by inactivating nuclear factor-kappaB and inhibiting mitogen-activated protein kinases. *International Journal of Molecular Medicine*, *30*(1), 204–210.

Perche, O., Vergnaud-Gauduchon, J., Morand, C., Dubray, C., Mazur, A., & Vasson, M. P. (2014). Orange juice and its major polyphenol hesperidin consumption do not induce immunomodulation in healthy well-nourished humans. *Clinical Nutrition: Official Journal of the European Society of Parenteral and Enteral Nutrition*, *33*(1), 130–135.

Protti, M., Valle, F., Poli, F., Raggi, M. A., & Mercolini, L. (2015). Bioactive molecules as authenticity markers of Italian Chinotto (Citrusxmyrtifolia) fruits and beverages. *Journal of Pharmaceutical and Biomedical Analysis*, *104*, 75–80.

Reynolds, P. R., Mucenski, M. L., & Whitsett, J. A. (2003). Thyroid transcription factor (TTF)-1 regulates the expression of

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midkine (MK) during lung morphogenesis. *Developmental Dynamics*, *227*(2), 227–237.

Saito, R. A., Watabe, T., Horiguchi, K., Kohyama, T., Saitoh, M., Nagase, T., & Miyazono, K. (2009). Thyroid transcription factor-1 inhibits transforming growth factor-beta-mediated epithelial-to-mesenchymal transition in lung adenocarcinoma cells. *Cancer Research*, *69*(7), 2783–2791.

Seyedrezazadeh, E., Kolahian, S., Shahbazfar, A. A., Ansarin, K., Pour Moghaddam, M., Sakhinia, M., Sakhinia, E., & Vafa, M. (2015). Effects of the flavanone combination hesperetin-naringenin, and orange and grapefruit juices, on airway inflammation and remodeling in a murine asthma model. *Phytotherapy Research: PTR*, *29*(4), 591–598.

Shi, Y., Dai, J., Liu, H., Li, R. R., Sun, P. L., Du, Q., Pang, L. L., Chen, Z., & Yin, K. S. (2009). Naringenin inhibits allergen-induced airway inflammation and airway responsiveness and inhibits NF-kappaB activity in a murine model of asthma. *Canadian Journal of Physiology and Pharmacology*, *87*(9), 729–735.

Takahashi, Y., Izumi, Y., Kohno, M., Kimura, T., Kawamura, M., Okada, Y., Nomori, H., & Ikeda, E. (2010). Thyroid transcription factor-1 influences the early phase of compensatory lung growth in adult mice. *American Journal of Respiratory and Critical Care Medicine*, *181*(12), 1397–1406.

Uchida, K., Shiraishi, M., Naito, Y., Torii, Y., Nakamura, Y., & Osawa, T. (1999). Activation of stress signaling pathways by the end product of lipid peroxidation. 4-Hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. *The Journal of Biological Chemistry*, *274*(4), 2234–2242.

Vanamala, J., Reddivari, L., Yoo, K. S., Pike, L. M., & Patil, B. S. (2006). Variation in the content of bioactive flavonoids in different brands of orange and grapefruit juices. *Journal of Food Composition and Analysis*, *19*(2–3), 157–166.

Yang, Y. L., Hsu, H. T., Wang, K. H., Han, C. Y., Chen, C. M., Chen, C. M., & Ko, W. C. (2011a). Hesperetin-7,3′-O-dimethylether selectively inhibits phosphodiesterase 4 and effectively suppresses ovalbumin-induced airway hyperresponsiveness with a high therapeutic ratio. *Journal of Biomedical Science*, *18*, 84.

Yang, Y., Wolfram, J., Shen, H., Fang, X., & Ferrari, M. (2012). Hesperetin: An inhibitor of the transforming growth factor-beta (TGF-beta) signaling pathway. *European Journal of Medicinal Chemistry*, *58*, 390–395. doi:10.1016/j.ejmech.2012.10.028.

Yang, Y., Xu, Y., Xia, T., Chen, F., Zhang, C., Liang, W., Lai, L., & Fang, X. (2011b). A single-molecule study of the inhibition effect of Naringenin on transforming growth factor-beta ligand-receptor binding. *Chemical Communications: Chem Comm/the Royal Society of Chemistry*, *47*(19), 5440–5442.

Zanotti Simoes Dourado, G. K., de Abreu Ribeiro, L. C., Zeppone Carlos, I., & Borges Cesar, T. (2013). Orange juice and hesperidin promote differential innate immune response in macrophages ex vivo. *International Journal for Vitamin and Nutrition Research. Internationale Zeitschrift fur Vitamin-Und Ernahrungsforschung. Journal International de Vitaminologie et de Nutrition*, *83*(3), 162–167.

Zhang, Y., Cardell, L. O., Edvinsson, L., & Xu, C. B. (2013). MAPK/NF-kappaB-dependent upregulation of kinin receptors mediates airway hyperreactivity: A new perspective for the treatment. *Pharmacological Research*, *71*, 9–18.

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