Citrus fruits are rich in flavonoids for immunoregulation and potential targeting ACE2

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Abstract

The most recent outbreak of 2019 novel coronavirus, named as COVID-19, caused pneumonia epidemic in Wuhan with 2,121 deaths cases as of February 20th 2020. Identification of effective antiviral agents to combat the novel coronavirus is urgently needed. Citrus fruit peel or wild citrus are rich in flavonoid, and is clinically documented for roles in relief of cough and promotion of digestive health. Therefore, citrus fruits are assumed to possess antivirus activities or enhance the host immunity. A previous study found that hesperetin could act as a high potent inhibitor of SARS-CoV 3CLpro. We determined six flavonoid compounds content of in three citrus species by using LC-MS technique. The content of naringin and naringenin was at higher levels in pummelo. Hesperetin and hesperidin were highly accumulated in mandarin and sweet orange. The subsequent *in vitro* and *in vivo* experiments indicated that naringin could inhibit the expression of the proinflammatory cytokines (COX-2, iNOS, IL-1β and IL-6) induced by LPS in Raw macrophage cell line, and may restrain cytokine through inhibiting HMGB1 expression in a mouse model. The results revealed that naringin may have a potential application for preventing cytokine storm. We simulated molecular docking to predict the binding affinity of those flavonoids to bind Angiotensin-converting enzyme 2 (ACE 2), which is a receptor of the coronavirus. Consideration of the potential anti-coronavirus and anti-inflammatory activity of flavonoids, the citrus fruit or its derived phytochemicals are promising in the use of prevention and treatment of 2019-nCoV infection.

Keywords: Citrus flavnoids, Naringin, Immunoregulation, ACE2, 2019-nCoV

Introduction

The number of pneumonia cases caused by a novel coronavirus (2019-nCoV) continues to rise in China. Thus far, there are tens of thousands of confirmed cases by the hospital agency. Common symptoms of patients infected with 2019-nCoV are fever, cough and myalgia or fatigue. The severe cases have high amounts of cytokines, such as TNF-α, IL-1β, IL-10, IFNγ and MCP-1, suggesting that the cytokine storm is associated with disease severity (Huang et al., 2020). Currently, there is no specific anti-viral treatment against the new coronavirus. Identifying effective antiviral agents to combat the disease is urgently needed. Commercial antiviral agents and chemical compounds extracted from traditional Chinese medicinal herbs were screened (Liu and Wang, 2020). Remdesivir and chloroquine were found to be highly effective in the control of 2019-nCoV infection in vitro (Wang et al., 2020). Some Chinese herbal compounds including baicalin, scutellarin, hesperetin, nicotianamine and glycyrrhizin were predicted to have a capacity for binding ACE2 with potential anti-2019-nCoV effects (Chen and Du, 2020). The existing safe host-directed therapies were repurposed to treat COVID-19 infection (Zumla et al., 2020). Traditional Chinese medicine was used to treat the novel coronavirus-infected pneumonia and proved to have a higher effect in the relief of cough and fever-reducing(Yao et al., 2020). In addition, corticosteroids were used frequently for severe cases treatment to reduce inflammatory-induced lung injury. Citrus is rich in bioactive compounds and some varieties are used as Chinese folk medicine, such as Zhiqiao and Zhishi (Sour orange, *Citrus aurantium*) or its hybrids, Huajuhong (*Citrus grandis*), and Chenpi (*Citris reticulata*). They have been clinically documented for roles in the relief of cough and the promotion of digestive health. Flavonoid compounds are expected to be developed as anti-viral drugs. Hesperetin was found the high potent inhibitor of SARS-CoV 3CLpro (Lin et al., 2005). Meanwhile, nutrient supplements could reduce the host immune responses. Therefore, we try to identify effective antiviral and anti-inflammation compounds from citrus flavonoids and give a nutritional recommendation for the prevention and treatment of COVID-19.

Results

Flavonoid profiling in citrus species

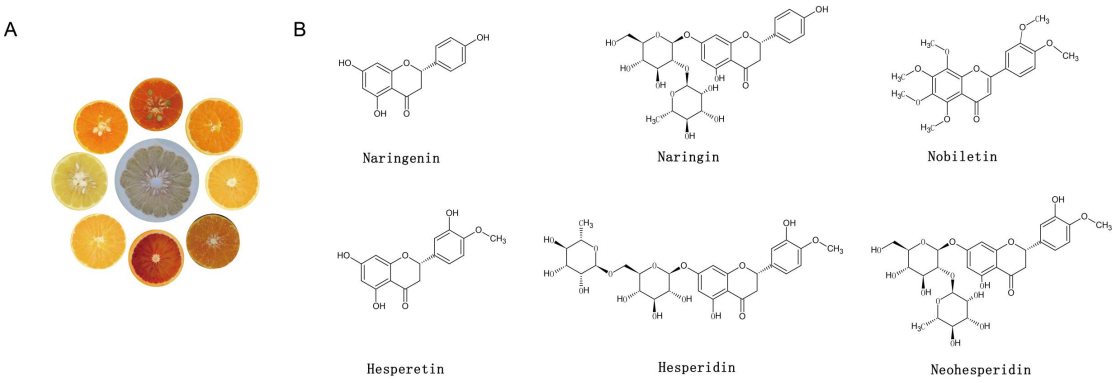

Figure 1. Citrus fruits (A) and chemical structure of naringenin, naringin, hesperetin, hesperidin, neohesperidin and nobiletin (B).

Citrus fruits are a rich source of vitamins, flavonoids and alkaloids. These phytochemicals have been reported to benefit human health, used to prevent and treat some diseases (Dall'Asta et al., 2013; Feng and Wang, 2018). To assess the potentials of six flavonoid compounds (Figure 1) variation in citrus, different cultivars were collected from three major species of mandarin (*Citrus reticulata*), pummelo (*Citrus maxima*) and sweet orange (*Citrus sinensis*) for targeted metabolic profiling. We have been detected and quantitated 459 known metabolites in the flesh, including segment membrane and juice sacs of 16 cultivars using LC-MS/MS. Among 459 known metabolites, we mainly analyzed the above six flavonoid compounds. The intensity of metabolic profiling signals for total ions reflected substantial qualitative and quantitative difference in different species. The relative content was represented by the average of relative contents of mandarins, pummelos, sweet oranges, respectively. Sixteen cultivars divided into mandarin, pummelo, sweet orange, were compared with each other. As showed in Figure 2A, the contents of naringin and naringenin were at higher levels in pummelo. On the other hand, mandarin and sweet oranges had higher hesperetin and hesperidin contents compared to pummelo. In general, the contents of neohesperidin and nobiletin were lower than the other four compounds in citrus.

Meanwhile, these six flavonoids were detected in selected 8 citrus cultivars, including ‘Kao Pan’ pummelo, ‘Majiayou’ pummelo, ‘Wanbai’ pummelo, ‘Oukan’ mandarin, Satsuma mandarin, Clementine mandarin, ‘Ponkan’ mandarin, ‘Newhall’ navel orange. Notably, the content of naringin was higher in ‘Kao Pan’ pummelo and ‘Wanbai’ pummelo than other cultivars. It was also found high content of naringin in herbal medicine Huajuhong. Hesperetin and hesperidin accumulated higher in ‘Ougan’, Satsuma mandarin and ‘Newhall’ navel orange. Herbal medicine Chenpi, Zhiqiao and Zhishi had high contents of these two compounds. Vitamins, rutin and herbal medicine Chenpi, Zhiqiao and Huajuhong were used in the treatment of 2019-nCoV infection. In order to prevent and decrease plasma cytokines levels of TNF-α, IL-1β, IL-10, IFNγ in COVID-19, citrus fruit or its derived phytochemicals should be an option to reduce the host immune responses and prevention of infection.

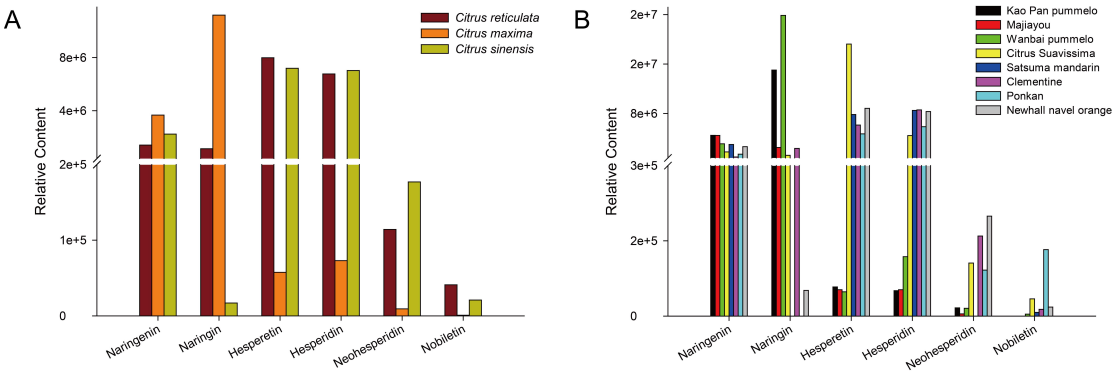

Figure 2. The content of six compounds were analyzed by LC-MS/MS (Shimadzu LCMS-8060) in different citrus species and cultivars. The data were analyzed by software, LabSolution Insight LCMS. Peak area of ions signal repreasent relative content. (A) Distribution of naringenin, naringin, hesperetin, hesperidin, neohesperidin and nobiletin in different citrus species. (B) The content of six flavonoid compounds in different cultivars.

Anti-inflammation of citrus naringin *in vitro* and *in vivo*

Cytokine storm was observed in most severe COVID-19 patients with increased plasma concentrations of TNF-α, IL-1β, IL-10, and IFNγ. Corticosteroids were used

frequently for severe cases of treatments to reduce inflammatory-induced lung injury. Therefore, treatment with anti-inflammatory approach is critical to alleviating clinical symptoms related to COVID-19. Lipopolysaccharide (LPS), a bacterial Gram-negative endotoxin, can induce cytokine storm with the increase of cytokines, such as IL-1β, TNF-α, IFNγ, IL-6 and MCP-1 (Ramos-Benitez et al., 2018). In addition, macrophage, one kind of the immune cells, can be caused by LPS to overzealously product inflammatory cytokines in immune response. The anti-inflammatory effect of citrus naringin (extracted from *Citrus wilsonii Tanaka*) on inflammatory cytokines, COX-2, iNOS, IL-1β and IL-6 were determined at mRNA levels in LPS-induced RAW 264.7 macrophages shown in Figure 3 A-D. The results demonstrated that the *COX-2*, *iNOS*, *IL-1β* and *IL-6* mRNA expression levels in LPS-treated macrophage cells were increased compared to the control group. Application of naringin (10, 20, 40 μg/mL) significantly diminished the effects of LPS induction of *COX-2*, *iNOS, IL-1β* and *IL-6* expression. Myocardial ischemia-reperfusion injury in rats were used to further examine the anti-inflammatory activity of naringin *in vivo*. The expression of high mobility group box 1 (HMGB1) level and the phosphorylation p38mitogen activated protein kinase (MAPK) level related to inflammation in the myocardium were measured to further investigate the anti-inflammatory activity of naringin. As shown in Figure 3 F-H, I/R caused a significant increase in expression levels of HMGB1, and p-p38 proteins compared to the Sham group. The naringin pretreatment significantly reversed the I/R effects on the expression of HMGB1 and p-p38 proteins.

Naringin exhibits a potent anti-inflammatory activity in the present and previous studies (Chtourou et al., 2016). It could inhibit the expression of the proinflammatory cytokines (COX-2, iNOS, IL-1β and IL-6) induced by LPS *in vitro* in the present study. Moreover, HMGB1 is a ubiquitous DNA-binding nuclear protein and can be released actively by immune cells, such as macrophages and monocytes, following inflammatory stimulation (Ulloa and Messmer, 2006; Wang et al., 2004). HMGB1 also acts as a pro-inflammatory cytokine and regulates cytokine storm, up-regulating cytokines such as TNF-α, IL-6, IL-1β, and IL-8 (Huang et al., 2010). It is further

demonstrated that naringin could restrain cytokine storm to a certain extent through inhibiting HMGB1 expression. Additionally, it was found that p38 MAPK has an important role in HMGB1-mediated production of proinflammatory cytokines (Park et al., 2003), and previous studies indicated inflammation is related to the phosphorylation level of p38 MAPK (Kim et al., 2019; Wu et al., 2019). Naringin pretreatment could attenuate p-p38 MAPK level in this study. These results revealed that naringin has potential use for preventing cytokine storm of COVID-19.

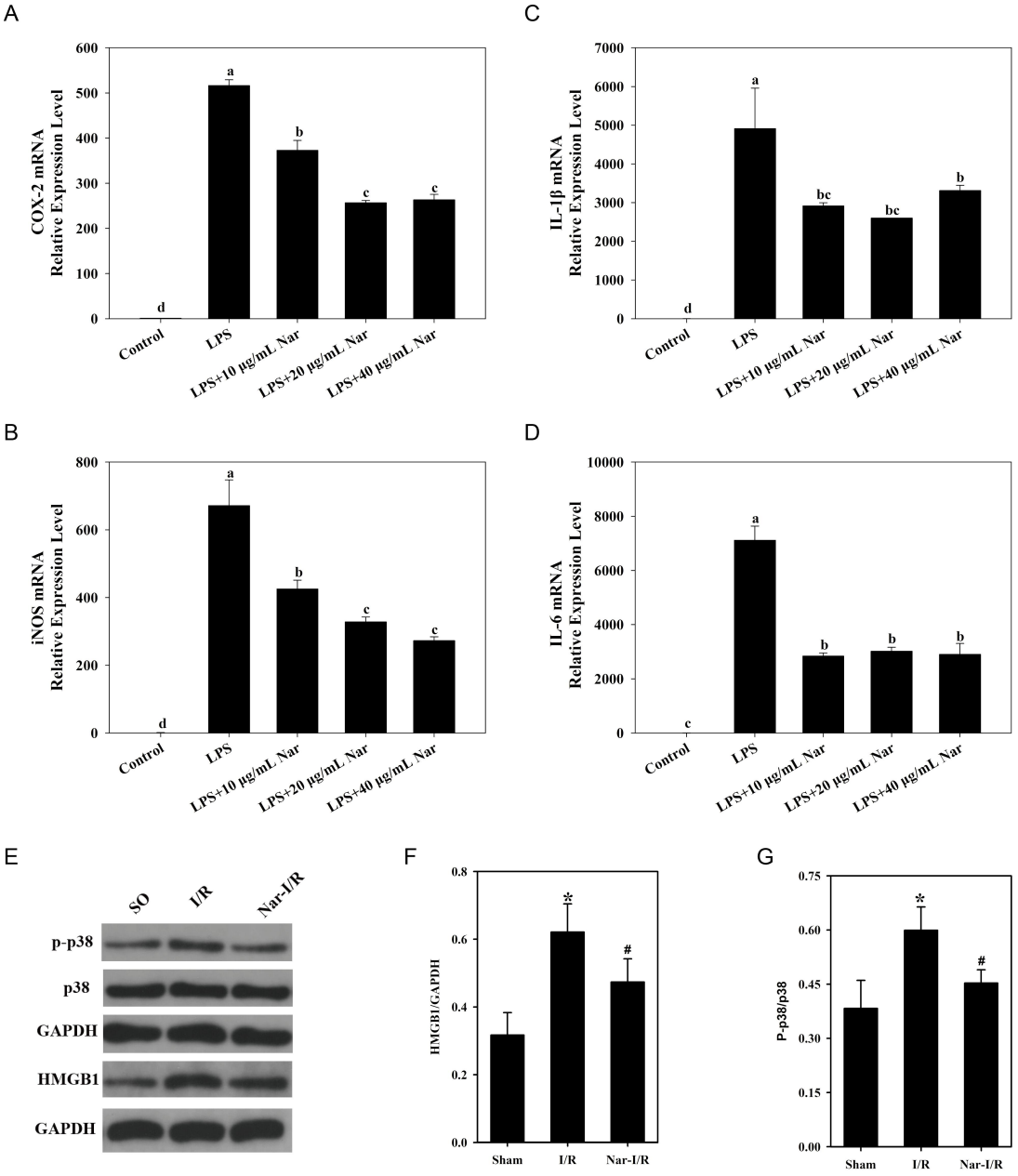

Figure 3. Anti-inflammation of naringin in vitro and in vivo. (A-D) Effect of naringin on LPS-induced mRNA expression of COX-2, iNOS, IL-1β, and IL-6 in RAW 264.7 macrophages.

The concentration of LPS is 1 μg/mL. (A) COX-2 mRNA expression; (B) iNOS mRNA expression; (C) IL-1β mRNA expression; (D) IL-6 mRNA expression. Different letters show significant differences ($p < 0.05$). (E-G) Anti-inflammation effect of naringin on myocardial ischemia/reperfusion injury in rats. Sham-operated control group (Sham): the rats were treated with solvent for naringin; Ischemia/reperfusion group (I/R): the rats were treated with solvent for naringin and subjected ischemia and reperfusion; Naringin + I/R group (Nar-I/R): the rats were treated with naringin (4 mg/kg per rat) and subjected ischemia and reperfusion. (E) Representative images of the Western blot results. (F) Expression levels of HMGB1 protein. (G) Expression levels of p-p38 protein. * $p < 0.05$ vs. the Sham group; # $p < 0.05$ vs. the I/R group.COX-2, cyclooxygenase-2; HMGB1, high mobility group box 1 protein; IL-1β, interleukin 1β; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion; LPS, lipopolysaccharide; Nar, naringin.

Molecular docking result of citrus flavonoids to ACE2 enzyme, a receptor of the coronavirus

In the last few weeks, rapid progress have been made in identification of viral etiology, since one genome sequence (WH-Human_1) of the 2019-nCoV released on Jan 10, 2020. Based on the computer-guided homology modeling method, it is found that 2019-nCoV S-protein and SARS-CoV S-protein shared an almost identical 3-D structure in the RBD domain and has a significant binding affinity to human ACE2 (Lu et al., 2020; Xu et al., 2020). ACE2 is widely expressed in the kidney, lung, brain, digestive tract and is considered to be critical for the coronavirus to enter host cells (Kuhn et al., 2006; Letko and Munster, 2020). Traditional herbal medicine was suggested to be promising for inhibiting coronavirus. Citrus showed broad pharmacological effects, including anti-obesity, anti-oxidant and anti-inflammation (Feng and Wang, 2018). To investigate whether citrus flavonoid compounds have the potential to anti-2019-nCoV, we simulated the molecular docking of the six compounds to predict their capacity for binding ACE2 (Figure 4). The interaction between citrus flavonoids and ACE2 were evaluated by binding energy. The docking

result showed that naringin may have highest binding activity to the ACE2 enzyme with estimated docking energy of -6.85 kcal/mol, with the potential binding site at TYR-515, GLU-402, GLU-398, and ASN394 (Figure 4A). Naringenin could bind to ACE2 with estimated docking energy of -6.05 kcal/mol, with binding site PRO-146, LEU-143, and LYS-131 (Figure 4B). As shown in Figure 4C, the stimulated result showed that hesperidin had the potential binding to ACE2 with docking energy of -4.21kcal/mol, with binding sites ASN-277, ARG-273, and HIS-505. The molecular docking of hesperetin to the ACE2 enzyme showed that hesperetin had the potential binding to ACE2 with docking energy of -6.09 kcal/mol, with binding sites LYS-562, GLU-564, GLY-205 (Figure 4D). Neohesperidin could bind to ACE2, with docking energy of -3.78 kcal/mol, with binding sites at TRP-349, ALA-348, TRP-69 (Figure 4E). Nobiletin could bind to ACE2 enzyme, with docking energy of -5.42 kcal/mol, and the potential binding site at TRP-69, LEU-351, ASP-350 (Figure 4F). These results suggested that among the six citrus flavonoids, the energy required for the binding between naringin and ACE2 was the lowest, followed by hesperetin, narigenin, indicating that they were easier to binding ACE2.

In addition, chloroquine and baicalin had been reported as potential inhibitors of SARS coronavirus infection in vitro test(Chen et al., 2004; Vincent et al., 2005). The cell surface expression of under-glycosylated ACE2 was inhibited by chloroquine, resulting in its poor affinity to SARS-CoV spike protein. We used molecular docking to calculate ACE2 binding energy of chloroquine and baicalin. As shown in Figure 4H, chloroquine had the potential binding to ACE2 with docking energy of -5.70 kcal/mol, and binding sites LEU-95, GLN-58, GLN-102. Baicalin could bind to ACE2 emzyme, with docking energy of -4.70 kcal/mol, with the potential binding site at HIS-374, HIS-378, and ALA-348 (Figure 4G). The docking energy of naringin, hesperetin and narigenin binding to ACE2 were comparable with chloroquine. It’s worthwhile to conduct further experiments to verify whether these citrus flavonoids could target ACE2 and prevent the infection of 2019-nCoV in cell culture models and laboratory animals.

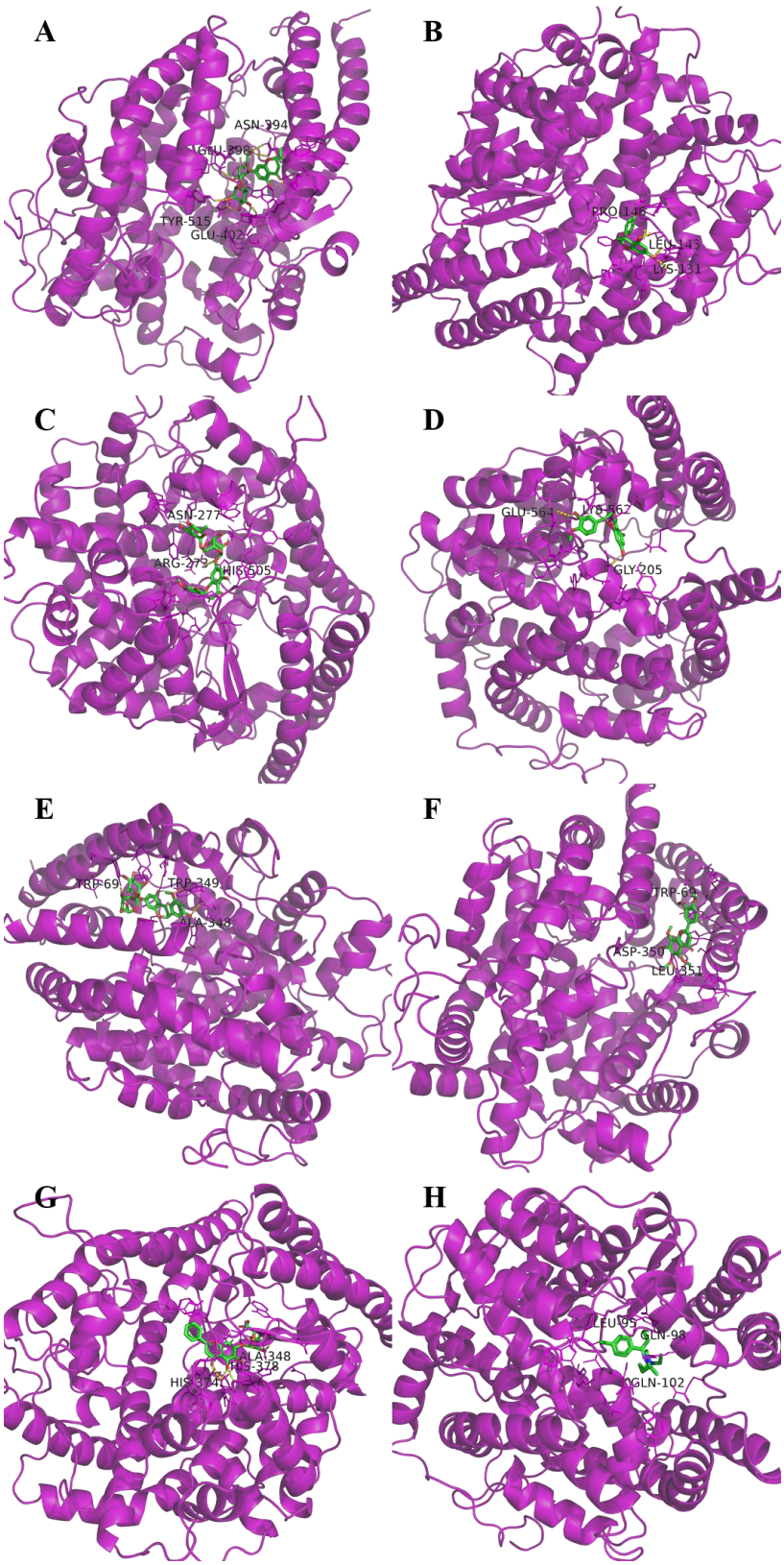

Figure 4. Molecular docking results of naringin (A), naringenin (B), hesperidin (C), hesperetin (D), neohesperidin (E), nobiletin (F), baicalin (G), and glycyrrhizin (H) to ACE2 enzyme (PDB code: 6ACG). The AutoDock 4.2 software was selected for the docking study using a hybrid Lamarckian Genetic Algorithm (LGA).

Conclusion

We determined the contents of six flavonoid compounds in three citrus species by using LC-MS technique. The contents of naringin and naringenin were at higher levels in pummelo. On the other hand, mandarin and sweet oranges had higher hesperetin and hesperidin contents compared to pummelo. The contents of neohesperidin and nobiletin were lower than other four compounds in citrus. Our data also showed that naringin could inhibit the expression of the proinflammatory cytokines (COX-2, iNOS, IL-1β and IL-6) induced by LPS *in vitro*. It was further demonstrated that naringin could restrain cytokine through inhibiting HMGB1 expression in a myocardial ischemic/reperfusion injury model. The results suggested that naringin could have a potential in preventing cytokine storms of COVID-19. The molecular docking result predicted that naringin and hesperetin had stronger binding affinity the ACE2. We suggested that these two phytochemicals, e.g., naringin and hesperetin are most potential compounds targeting ACE2 receptor, which could prevent coronavirus infection. Chinese traditional medicine is playing an important role in the treatment of COVID-19. We should pay more attention to natural compounds form citrus and other herbal medicine to combat coronavirus in the future.

Funding

The study was supported by the National Key Research and Development Program (2018YFD1000200), the Ability Establishment of Sustainable Use for Valuable Chinese Medicine Resources (2060302).

Acknowledgments

We thank Dr. Shan Li, Dr. Guiqing Peng, Dr. Ping Yin for their good advice. We thank Dr. Dingbo Lin for editing the manuscript. Wuhan fighting.

Conflict of interest

The authors declare no conflict of interest.

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