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REVIEW

Coronavirus enzyme inhibitors-experimentally proven natural compounds from plants

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Coronavirus disease (COVID-19) can cause critical conditions that require efficient therapeutics. Several medicines are derived from plants, and researchers are seeking natural compounds to ameliorate the symptoms of COVID-19. Viral enzymes are popular targets of antiviral medicines; the genome of coronaviruses encodes several enzymes, including RNA-dependent RNA polymerase and viral proteases. Various screening systems have been developed to identify potential inhibitors. In this review, we describe the natural compounds that have been shown to exert inhibitory effects on coronavirus enzymes. Although computer-aided molecular structural studies have predicted several antiviral compound candidates, the current review focuses on experimentally proven natural compounds.

Keywords: coronavirus, COVID-19, SARS-CoV-2, natural compounds

Introduction

The Spanish flu pandemic caused 50–100 million deaths worldwide in 1918–1919; approximately 100 years later, the coronavirus disease (COVID-19) pandemic has caused over 4 million deaths in 2019–2021 (Aassve *et al.*, 2021). Although several COVID-19 vaccines have been developed, the appearance of mutant forms has prevented the eradication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by vaccination (Lopez Bernal *et al.*, 2021; Tregoning *et al.*, 2021). Therefore, the development of antiviral drugs for COVID-19 is urgently needed, and many researchers are seeking medicines that are effective against the disease (Ghosh *et al.*, 2020; Khare *et al.*, 2020). As coronaviruses have been responsible for occasional epidemics causing mild upper respiratory tract illness, SARS-CoV-2 has been predicted to co-exist with humans for an extended period of time (Rucinski *et al.*, 2020). For this reason, alternative medicines that can alleviate the symptoms of COVID-19 will be welcomed (Nugraha *et al.*, 2020). In this review, we describe natural compounds that have shown an inhibitory effect on coronavirus enzymes. These natural compounds are potentially effective in alleviating COVID-19 symptoms by inhibiting coronavirus infection and replication.

The genomes of most viruses encode enzymes that enable viral replication, and these enzymes are employed as targets for antiviral drugs. The genome of herpesviruses, for example, encodes thymidine kinase, and drugs such as acyclovir have been developed to target thymidine kinase (Pallasch *et al.*, 1984; de Clercq, 1993). The human immunodeficiency virus (HIV) genome encodes reverse transcriptase and protease, and many antiviral drugs have been developed using these enzymes as targets (Collier *et al.*, 1996; Staszewski *et al.*, 1999). Similarly, the hepatitis C virus genome encodes RNA polymerase and protease, and the combination of these inhibitors can be used to treat HCV infection (Koev *et al.*, 2007; Kwong *et al.*, 2008). Finally, the genome of influenza virus encodes neuraminidase, which is required for virus release, and oseltamivir and zanamivir are popular medicines for the treatment of influenza-related diseases (Jackson *et al.*, 2011; Abed and Boivin, 2017). Coronavirus enzymes are thus popular targets for coronavirus treatment, and many researchers are seeking coronavirus medicines using these enzymes. However, clinically effective inhibitors are not currently available (Pawar, 2020). Natural compounds, especially plant-derived phytochemicals, have a long history of application as a traditional medicine for human health, and many medicines have been derived from natural compounds (Cragg, 2002). Because herbal medicines have been used for a long time, these natural compounds are expected to have relatively low toxicity (Ruhul Amin *et al.*, 2009).

Methods

This review deals with natural compounds that have shown an inhibitory effect on coronavirus enzymes. Although many studies have predicted the potential inhibitory effects of natural compounds on coronavirus enzymes using computer-based simulations such as docking studies, simulation predictions often differ from experimental results. For example, lopinavir-ritonavir was believed to be effective against SARS-CoV-2 3CL-protease; however, experimental results and cli-

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nical studies have shown that lopinavir-ritonavir is effective against neither coronavirus enzymes nor COVID-19 (Nukoolkarn *et al.*, 2008; Cao *et al.*, 2020; Jang *et al.*, 2020b; Ortega *et al.*, 2020; Stower, 2020). For this reason, this review focuses on research results that include laboratory experimental data. In addition, the review excludes the results of natural compound inhibitors with very high half maximal inhibitory concentration (IC_{50}) values (IC_{50} > 100 μM).

Coronavirus Enzymes

Among the four coronavirus subfamilies (alpha, beta, gamma, and delta), alpha and beta coronavirus subfamilies are known to infect humans (Velavan and Meyer, 2020). SARS-CoV-2, Middle East Respiratory Syndrome (MERS), and SARS-CoV belong to the beta subfamily and cause severe diseases. There are four additional coronaviruses that infect humans, namely,

Table 1. Natural compounds targeting coronavirus 3CL-protease

Botanical name	Plant part	Chemical name	Virus	IC_{50} (μM)	Reference
Anacardium occidentale	fruit	anacardic acids	SARS-CoV-2	2.07	Chen *et al.* (2021)
Angelica keiskei	whole	isobavachalcone	SARS-CoV	39.4	Park *et al.* (2016)
		xanthoangelol		38.4	
		xanthoangelol F		34.1	
		xanthoangelol D		26.6	
		xanthoangelol E		11.4	
		xanthoangelol B		22.2	
		xanthokeistal A		44.1	
Betula pubescens	bark	betulinic acid	SARS-CoV	10	Wen *et al.* (2007)
Camellia sinensis	leaf	epigallocatechin gallate (EGCG)	SARS-CoV-2	16.5	Jang *et al.* (2020a)
				4.24	Chiou *et al.* (2021)
				7.51	Zhu and Xie (2020)
				0.874	Du *et al.* (2021)
			SARS-CoV	24.98	Chiou *et al.* (2021)
				>100	Chen *et al.* (2005)
				73	Nguyen *et al.* (2012)
			HCoV-OC43	31.8	Jang *et al.* (2021)
			HCoV-229E	25.5	Jang *et al.* (2021)
		theaflavin	SARS-CoV-2	14.9	Jang *et al.* (2021)
			SARS-CoV	56	Chen *et al.* (2005)
		theaflavin-3, 3′-digallate	SARS-CoV	9.5	Chen *et al.* (2005)
Cirsium setidens	flower	pectolinarin	SARS-CoV-2	51.64	Jo *et al.* (2020b)
			SARS-CoV	37.78	Jo *et al.* (2020a)
Ginkgo biloba	leaf	ginkgolic acid	SARS-CoV-2	1.79	Chen *et al.* (2021)
		ginkgolic acid		0.7~3.57	Xiong *et al.* (2021)
		genkwanin		10.62	
		quercetin		12.65	
		isorhamnetin		31.59	
		luteolin		74.86	
		apigenin		84.94	
		sciadopitysin		1.09	
		ginkgetin		2.98	
		isoginkgetin		2.33	
		amentoflavone		8.65	
		bilobetin		11.19	
Isatis indigotica	root	hespretin	SARS-CoV	8.3	Lin *et al.* (2005)
Pistacia lentiscus	leaf	1,2,3,4,6-pentagalloylglucose	SARS-CoV-2	3.66	Chiou *et al.* (2021)
Pterocarpus santalinus	root	savinin	SARS-CoV	25	Wen *et al.* (2007)
Rhodiola rosea	root	herbacetin	SARS-CoV-2	53.90	Jo *et al.* (2020b)
			SARS-CoV	33.17	Jo *et al.* (2020a)
Rhus semialata	fruit	tannic acid	SARS-CoV-2	13.4	Wang *et al.* (2020)
Rhus succedanea	leaf	rhoifolin	SARS-CoV	27.45	Jo *et al.* (2020a)
Scutellaria baicalensis	root	baicalin	SARS-CoV-2	34.71	Jo *et al.* (2020b)
				6.41	Su *et al.* (2020b)
		baicalein		0.94	Su *et al.* (2020b)
		Scutellarin		3.02	Su *et al.* (2020b)

human coronavirus OC43 (HCoV-OC43), HCoV-HKU1, HCoV-229E, and HCoV-NL63 (Rucinski *et al.*, 2020). HCoV-229E and CoV-NL63 belong to the alpha coronavirus subfamily, and HCoV-OC43 and HCoV-HKU1 belong to the beta coronavirus subfamily (Bahadur *et al.*, 2020). Although these coronaviruses share significant homology with SARS-CoV-2, they cause mild symptoms. They are thus suitable as model viruses to study SARS-CoV-2 under less strict conditions (Jang *et al.*, 2021).

The genome of coronaviruses, including SARS-CoV-2, encodes several essential enzymes that are required for their replication (Thiel *et al.*, 2003; Ziebuhr, 2004). These enzymes are therefore employed as drug targets for the discovery of novel coronavirus drugs. The most popular enzymes are RNA-dependent RNA polymerase (RdRp) and proteases (Ullrich and Nitsche, 2020; Zhu *et al.*, 2020). Because coronaviruses have a single-stranded positive-sense RNA genome (Baltimore class III), their genome encodes an RNA-dependent RNA polymerase for their replication (Gao *et al.*, 2020). As the structure of RNA-dependent RNA polymerase is distinct from that of cellular DNA-dependent RNA polymerase, RNA-dependent RNA polymerase is a suitable target for coronavirus drug development (Zhu *et al.*, 2020). Other popular targets are coronavirus proteases. The genomes of many viruses encode polyproteins to minimize the genome size, and these polyproteins are cleaved into individual functional proteins by virus-encoded proteases (Anderson *et al.*, 2009). These viral proteases are therefore essential for virus replication, and specific inhibitors of these proteases are developed into antiviral drugs (Kim *et al.*, 2013). Coronavirus genomes encode two viral proteases, a 3C-like protease (3CL-pro) and a papain-like protease (PL-pro). Because 3CL-pro has 11 cleavage sites in the coronavirus polyprotein and PL protease has four, 3CL-pro is also known as the main protease (Mpro) (Anand *et al.*, 2003; Hsu *et al.*, 2005). Recently, several additional enzymes, such as uridylate-specific endoribonuclease, have been proposed as drug targets for coronavirus therapy (Hong *et al.*, 2021).

3CL-protease (3CL-pro)

3CL-pro is an essential enzyme that cleaves the coronavirus polyprotein at multiple sites; therefore, the inhibition of 3CL-pro also inhibits the replication of coronaviruses (Anand *et al.*, 2003; Hsu *et al.*, 2005). For example, peptide-based competitive inhibitors were tested as inhibitors of SARS-CoV-2 3CL-pro and were found to significantly inhibit SARS-CoV-2 replication (Zhang *et al.*, 2020).

Protease assays are relatively easy to perform using fluorescence resonance energy transfer (FRET)-based systems, and many natural compounds have been thus tested to examine their inhibitory activity (Jo *et al.*, 2020a; Khare *et al.*, 2020). Although many computer-based simulations or docking studies have been performed, we focused on experimental data in this review. Various natural compounds have been identified as inhibitors of 3CL-pro, and assays are usually performed with SARS-CoV-2 and SARS-CoV (Table 1). IC_{50} was determined using the 3CL-pro assay and can be used to estimate the relative inhibitory activity.

Extensive studies have been performed with tea catechins, and epigallocatechin gallate (EGCG) and theaflavin have been reported to be effective inhibitors of coronavirus 3CL-pro (Chen *et al.*, 2005; Park *et al.*, 2021). Multiple studies have shown that EGCG inhibits SARS-CoV-2 3CL-pro, and the IC_{50} range of EGCG was found to be 0.847–16.5 μM (Jang

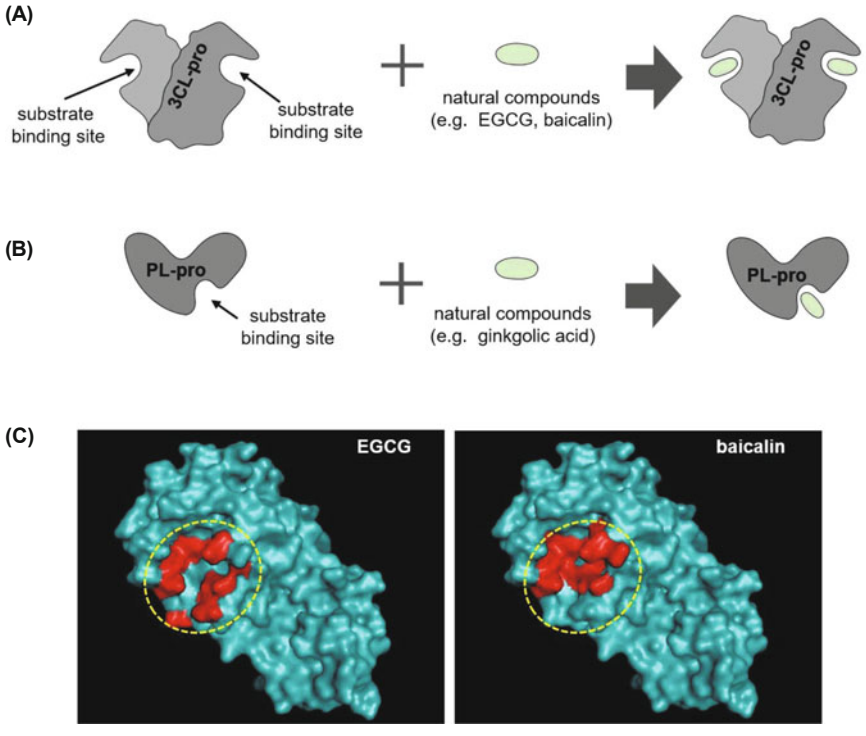

Fig. 1. *In silico* molecular docking study revealed the potential binding modes of natural compounds to coronavirus proteases. Natural compounds occupy the substrate binding site of coronavirus proteases. (A) 3CL-pro, (B) PL-pro. (C) 3CL-pro structure is shown in cyan, and the potential binding sites of EGCG and baicalin are shown in red. Yellow circle indicates the substrate binding site.

et al., 2020a; Chiou *et al.*, 2021; Du *et al.*, 2021). Interestingly, EGCG has been reported to be more effective against SARS-CoV-2 than against SARS-CoV and other human coronaviruses (Chiou *et al.*, 2021; Park *et al.*, 2021). In one study, EGCG was reported to be ineffective against SARS-CoV 3CL-pro, with an IC_{50} of over 100 μM (Chen *et al.*, 2005). However, treatment with EGCG has been shown to inhibit the replication of coronavirus in infected cells (Hong *et al.*, 2021; Jang *et al.*, 2021). Because green tea contains a high percentage of EGCG, the relationship between consumption of green tea and COVID-19 morbidity/mortality was studied through an examination of statistical data, and countries with high green tea consumption showed relatively low morbidity/mortality due to COVID-19 (Storozhuk, 2020). Recently, a preliminary clinical study was performed to demonstrate the efficacy of green tea in COVID-19 patients (Bettuzzi *et al.*, 2021).

To identify potential SARS-CoV-2 inhibitors, high-throughput screening of 1920 natural products was performed using SARS-CoV-2 proteases, and ginkgolic acid from *Ginkgo biloba* and anacardic acid from *Anacardium occidentale* were identified as effective inhibitors (Chen *et al.*, 2021). Interestingly, both ginkgolic acid and anacardic acid exerted inhibitory effects on both 3CL-pro and PL-pro, and the IC_{50} value for 3CL-pro was found to be lower than the IC_{50} of PL-

Table 2. Natural compounds targeting coronavirus PL-protease

Botanical name	Plant part	Chemical name	Virus	IC_{50} (μM)	Reference
Alnus japonica	bark	hirsutenone	SARS-CoV	4.1	Park *et al.* (2012)
		hirsutanonol		7.8	
		oregonin		20.1	
		rubranol		12.3	
		rubranoside B		8.0	
		rubranoside A		9.1	
Anacardium occidentale	fruit	anacardic acids	SARS-CoV-2	17.08	Chen *et al.* (2021)
Angelica keiskei	leaf	isobavachalcone	SARS-CoV	13	Park *et al.* (2016)
		4-hydroxyderricin		26	
		xanthoangelol		11.7	
		xanthoangelol F		5.6	
		xanthoangelol D		19.3	
		xanthoangelol E		1.2	
		xanthoangelol B		11.7	
		xanthoangelol G		46.4	
		xanthokeistal A		21.1	
Curcuma longa	root	curcumin	SARS-CoV	5.7	Park *et al.* (2012)
Ginkgo biloba	leaf	Ginkgolic acid	SARS-CoV-2	16.30	Chen *et al.* (2021)
Paulownia tomentosa	fruit	tomentin A	SARS-CoV	6.2	Cho *et al.* (2013)
		tomentin B		6.1	
		tomentin C		11.6	
		tomentin D		12.5	
		tomentin E		5.0	
		3′-*O*-methyldiplacol		9.5	
		4′-*O*-methyldiplacol		9.2	
		3′-*O*-methyldiplacone		13.2	
		4′-*O*-methyldiplacone		12.7	
		mimulone		14.4	
		diplacone		10.4	
		6-geranyl-4′,5,7-trihydroxy-3′,5′-dimethoxyflavanone		13.9	
Psoralea corylifolia	seed	bavachinin	SARS-CoV	38.4	Kim *et al.* (2014)
		neobavaisoflavone		18.3	
		isobavachalcone		7.3	
		4′-O-methylbavachalcone		10.1	
		psoralidin		4.2	
		corylifol A		32.3	
Tribulus terrestris	fruit	*N-trans*-caffeoyltyramine	SARS-CoV	44.4	Song *et al.* (2014)
		N-trans-coumaroyltyramine		38.8	
		N-trans-feruloyltyramine		70.1	
		terrestriamide		21.5	
		N-trans-feruloyloctopamine		26.6	
		terrestrimine		15.8	

pro, indicating that 3CL-pro is more susceptible to ginkgolic acid and anarcardic acid treatment (Chen *et al*., 2021). In addition, both ginkgolic acid and anacardic acid were effective in reducing the number of SARS-CoV-2 induced plaques (Chen *et al*., 2021). In another study, a screening of 80 herbal compounds showed that ginkgo extract was effective in inhibiting SARS-CoV-2 3CL-pro, and ginkgolic acid and other natural compounds from ginkgo leaves were identified as the compounds responsible for this inhibition (Xiong *et al*., 2021).

In a screening of 70 flavonoids, baicalin was identified as an effective inhibitor of SARS-CoV-2 3CL-pro (Jo *et al*., 2020b). A separate study also revealed that baicalin and baicalein from *Scutellaria baicalensis* showed inhibitory activity against SARS- CoV-2 3CL-pro (Su *et al*., 2020b). Moreover, baicalin and baicalein have been found to show inhibitory activity against SARS-CoV-2 RNA-dependent RNA polymerase (Zandi *et al*., 2021).

Coronavirus 3CL-pro has a substrate binding pocket and catalytic residues, and *in silico* molecular docking analysis predicted that the natural compounds such as EGCG, ginkgolic acid and baicalin occupy the substrate binding site by forming hydrogen bonds with multiple residues of 3CL-pro (Su *et al*., 2020; Chen *et al*., 2021; Chiou *et al*., 2021; Iketani *et al*., 2021). These *in silico* analysis results provide the possible explanation for how the natural compounds inhibit coronavirus 3CL-pro (Fig. 1A and C).

Papain-Like protease (PL-pro)

The coronavirus genome encodes papain-like protease (PL-pro), which cleaves the coronavirus polyprotein (Klemm *et al*., 2020). PL-pro is also known to remove ubiquitin-like ISG15 protein and Lys48-linked polyubiquitins (Freitas *et al*., 2020). Because coronavirus PL-pro is an essential enzyme, PL-pro is regarded as an important antiviral target (Klemm *et al*., 2020). There are several natural compounds that have shown an inhibitory effect on the PL-pro of coronaviruses such as SARS-CoV (Table 2). Currently, the number of publications on SARS-CoV-2 PL-pro is less than that on 3CL-pro; because 3CL-pro is regarded as the main protease, initial studies were performed with 3CL-pro.

Alnus japonica is a popular traditional medicine in Korea for the treatment of cancer and hepatitis (Kim *et al*., 2004; Sati *et al*., 2011). The ethanol extract of air-dried *Alnus japonica* bark was used to identify PL-pro inhibitors, and the purified diarylheptanoid exerted an inhibitory effect on SARS-CoV PL-pro (Park *et al*., 2012).

Angelica keiskei mainly grows along the Pacific coast of Japan, where it is also used as a diuretic and tonic (Akihisa *et al*., 2003). An ethanol extract of *Angelica keiskei* showed 75% inhibition of SARS-CoV 3CL-pro and 88% inhibition of PL-pro at 30 μg/ml (Park *et al*., 2016). Compounds isolated from *Angelica keiskei* also exerted significant inhibitory effects on both 3CL-pro and PL-pro (Park *et al*., 2016).

Tribulus terrestris is distributed throughout India and the southern part of China, and its fruits are widely used in pharmaceutical preparations and food supplements (Kostova and Dinchev, 2005). A methanol extract of *Tribulus terrestris* fruit showed potent inhibition of SARS-CoV PL-pro, and bioactivity-guided purification resulted in the identification of cinnamic amide and ferulic acid as natural compound PL-pro inhibitors (Song *et al*., 2014).

Paulownia tomentosa is a polyphenol-rich plant that has been used in Chinese traditional medicines (Cho *et al*., 2013). Consumption of *Paulownia tomentosa* fruit is known to relieve bronchitis, especially by reducing coughing and asthma (Schneiderová and Šmejkal, 2015). A methanol extract of *Paulownia tomentosa* fruit was purified to yield natural compounds that showed inhibitory effects on PL-pro, and five novel geranylated flavonoids (tomentin A, tomentin B, tomentin C, tomentin D, and tomentin E) were identified as natural compound inhibitors of SARS-CoV PL-pro (Cho *et al*., 2013).

Coronavirus PL-pro also has a substrate binding site like 3CL-pro, and *in silico* molecular docking analysis showed the substrate binding pocket was occupied by the natural compounds including ginkgolic acid and xanthoangelol (Park *et al*., 2016; Chen *et al*., 2021). These results suggest that the interaction of natural compounds with the substrate binding site of PL-pro contributes to the inhibition of coronavirus PL-pro (Fig. 1B).

RNA-Dependent RNA Polymerase and Other Targets

Coronaviruses have a single-stranded RNA genome and require RNA-dependent RNA polymerase for replication (Xu *et al*., 2003). Because several viral RNA-dependent RNA polymerase inhibitors have been developed, these inhibitors have been tested to treat coronavirus diseases (Furuta *et al*., 2013; Tchesnokov *et al*., 2019). Clinical results showed that ribavirin and remdesivir have no or limited effects in the treatment of COVID-19 (Beigel *et al*., 2020; Hung *et al*., 2020; Tong *et al*., 2020; Young *et al*., 2021).

Although coronavirus RNA-dependent RNA polymerase is an attractive target for drug development, the screening system is not as simple as other protease systems (Zandi *et al*., 2021). For this reason, many inhibitors were tested using *in-silico* methods; however, few studies were performed to screen coronavirus RNA-dependent RNA polymerase. Baicalein and baicalin from *Scutellaria baicalensis* showed inhibitory effects on coronavirus RNA-dependent RNA polymerase (Zandi *et al*., 2021). Baicalein and baicalin also exerted an inhibitory effect on the replication of other RNA viruses, including dengue virus and chikungunya virus (Zandi *et al*., 2012; Moghaddam *et al*., 2014; Lani *et al*., 2016).

The SARS-CoV-2 genome encodes uridylate-specific endoribonuclease (non-structural protein 15, Nsp15), and Nsp15 cleaves poly U sequences of viral RNA intermediates to decrease the level of poly U-containing sequences (Ulferts and Ziebuhr, 2011; Kim *et al*., 2020). The host innate immune system can recognize the poly U sequence in the viral genome; therefore, Nsp15 can help the virus to evade the host innate immune system (Kindler *et al*., 2017; Hackbart *et al*., 2020). EGCG and baicalin have been reported to inhibit the function of Nsp15, and the inhibition of Nsp15 appears to contribute to the inhibition of coronavirus replication (Hong *et al*., 2021).

Discussion

In this review, we summarize the experimental results of coronavirus enzyme inhibitors derived from plants. Because protease assays are relatively easy to perform, many inhibitors have been discovered using coronavirus 3CL-pro and coronavirus PL-pro. Several reports have shown that inhibition of coronavirus replication in cultured cells indicates that these enzyme inhibitors are effective in reducing coronavirus replication in vitro (Chen *et al*., 2021; Hong *et al*., 2021; Jang *et al*., 2021). Because an effective medicine against coronavirus is not available, an approach with natural compounds will be useful in developing effective coronavirus therapies.

Among the list of coronavirus enzyme inhibitors, the efficacy of several compounds was confirmed in multiple studies, and these repeated results make the data more reliable. EGCG, theaflavin, ginkgolic acid, and baicalin have been confirmed as effective inhibitors (Jang *et al*., 2020a; Jo *et al*., 2020b; Chen *et al*., 2021). In addition, several compounds, including ginkgolic acid, anacardic acid, baicalin, and EGCG, target multiple coronavirus enzymes. Further studies should be conducted to determine the major target of these compounds. For example, ginkgolic acid and anacardic acid appear to be more effective against SARS-CoV-2 3CL-pro than against SARS-CoV-2 PL-pro because the IC_{50} of 3CL-pro is notably lower than the IC_{50} of PL-protease (Chen *et al*., 2021). However, the effect of each enzyme inhibition on the replication of the virus is different, and the inhibitory effect on coronavirus replication cannot be judged simply by the scale of IC_{50}.

Because herbal medicine has been used for a long time, natural compounds from plants or plant extracts are considered relatively safe. For example, green tea has been consumed for thousands of years, and green tea contains a relatively high percentage of EGCG (Park *et al*., 2021). In the future, preclinical trials including animal experiments and clinical trials should be conducted to validate the efficacy of these natural compounds *in vivo*.

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Conflict of Interest

The authors have no conflict of interest to report.

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