

# BPB Reports

## Report

# Inhibitory Effects of Olive-Derived Phytochemicals on SARS-CoV-2 3C-Like Protease (3CL<sup>pro</sup>)

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus that caused the global epidemic of COVID-19. 3C-like protease (3CL<sup>pro</sup>), called the main protease (M<sup>pro</sup>), cleaves 11 sites in the long-stranded protein synthesized from viral genomic RNA. After the cleavage of the long-stranded protein, proteins such as RNA-dependent RNA polymerase are cut off and activated. Therefore, 3CL<sup>pro</sup> is crucial for SARS-CoV-2 replication and is the target of drugs against SARS-CoV-2. Polyphenols and triterpenoids in olives can bind to SARS-CoV-2 3CL<sup>pro</sup> in *in silico* and computational studies. However, not all phytochemicals have been shown to inhibit SARS-CoV-2 3CL<sup>pro</sup>. Here, we examined the inhibitory effects of olive-derived phytochemicals on SARS-CoV-2 3CL<sup>pro</sup>. Among these phytochemicals, luteolin and oleanolic acid inhibited the 3CL<sup>pro</sup> activity by about 40%. The actual enzyme inhibitory action of oleanolic acid has not been examined. Thus, we examined the dose response in the inhibition of 3CL<sup>pro</sup> by oleanolic acid. Oleanolic acid significantly inhibited the enzymatic activity of 3CL<sup>pro</sup> at concentrations above 50  $\mu$ M, and it may be a potent molecule that inhibit SARS-CoV-2 replication via 3CL<sup>pro</sup> inhibition.

**Key words** olive, oleanolic acid, SARS-CoV-2, COVID-19, 3C-like protease, 3CL<sup>pro</sup>, main protease, M<sup>pro</sup>

## INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus that caused the global epidemic of COVID-19. SARS-CoV-2 has a genomic RNA of approximately 30,000 bases, which is the basis for producing several proteins necessary for replication and infection.<sup>1,2)</sup> These proteins are structural proteins, such as spike glycoproteins and envelopes, and nonstructural proteins (NSPs), such as proteases and RNA-dependent RNA polymerase (RdRP). 3C-like protease (3CL<sup>pro</sup>, NSP5) and papain-like protease (PL<sup>pro</sup>, NSP3) cleave 11 and 3 sites in the long-stranded protein synthesized from viral genomic RNA, respectively. Thus, 3CL<sup>pro</sup> is also called the main protease (M<sup>pro</sup>). In particular, 3CL<sup>pro</sup> cleaves the SARS-CoV-2 protein to generate RdRP (NSP12), NSP7, and NSP8, which form a complex to copy the genomic RNA of SARS-CoV-2.<sup>3)</sup> Therefore, 3CL<sup>pro</sup> as well as RdRP is crucial for SARS-CoV-2 replication, and 3CL<sup>pro</sup> is the target for drugs against SARS-CoV-2.<sup>4,5)</sup>

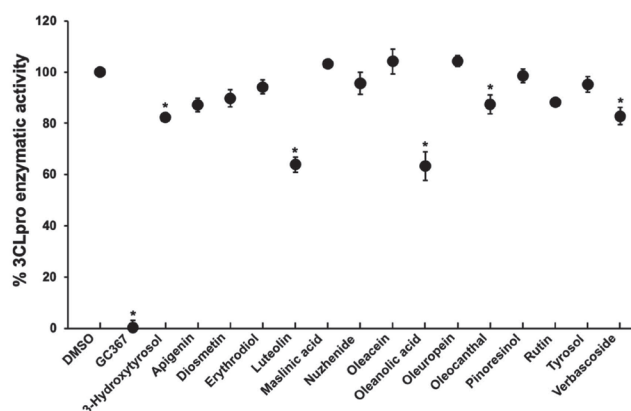
Various phytochemicals, including polyphenols and triterpenoids, have been isolated from olive leaves and fruits. A recent report has shown that polyphenols and triterpenoids in olives, such as oleuropein and oleanolic acid, can bind to SARS-CoV-2 3CL<sup>pro</sup> in *in silico* and computational studies.<sup>6)</sup> However, not all polyphenols and terpenoids, which bind to 3CL<sup>pro</sup> in *in silico* studies, have been shown to inhibit 3CL<sup>pro</sup>. Here, we examined the inhibitory effects of olive-derived phytochemicals on SARS-CoV-2 3CL<sup>pro</sup>.

## MATERIALS AND METHODS

**Materials** The 3CL protease (SARS-CoV-2) assay kit (Cat# 78042-1) was obtained from BPS Bioscience Inc. (San Diego, CA, USA). Oleocanthal was obtained from Merck (Kenilworth, NJ, USA). Diosmetin, luteolin, oleanolic acid, oleuropein, and verbascoside were obtained from Cayman Chemical (Ann Arbor, MI, USA). Apigenin, 3-hydroxytyrosol (3, 4-Dihydroxyphenyl ethanol), diosmetin, erythrodiol, tyrosol (p-hydroxyphenylethanol), maslinic acid, pinoresinol, rutin, and dimethyl sulfoxide (DMSO) for molecular biology were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Nuzhenide was obtained from Selleck Chemicals (Houston, TX, USA). Oleacein was obtained from Toronto Research Chemicals (Toronto, ON, Canada).

**3CL<sup>pro</sup> Activity** The 3CL<sup>pro</sup> activity assay was performed using a 3CL protease (SARS-CoV-2) assay kit according to the manufacturer's instructions. The final concentration of phytochemicals in the reaction mixture in the assay was 50  $\mu$ M, and all phytochemicals were dissolved in DMSO.<sup>7)</sup> GC376 at 100  $\mu$ M was used as the inhibitor control.<sup>8)</sup> The fluorescence emitted when the substrate is cleaved by 3CL<sup>pro</sup> is an excitation light at 360 nm and an emission light at 460 nm and the fluorescence intensity was measured by SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA). Wells containing substrate, DMSO (1%), and enzymes were used as positive controls. As a blank, wells containing substrate, DMSO (1%), and no enzyme were used. The blank val-

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**Fig. 1.** The Inhibitory Effects of Olive-Derived Phytochemicals (50  $\mu\text{M}$ ) on SARS-CoV-2 3CL<sup>pro</sup>

3CL<sup>pro</sup> inhibitory activity observed in the DMSO-treated control was 100%. Values are expressed as means  $\pm$  SEM (n = 3).  $p < 0.05$  was considered statistically significant.

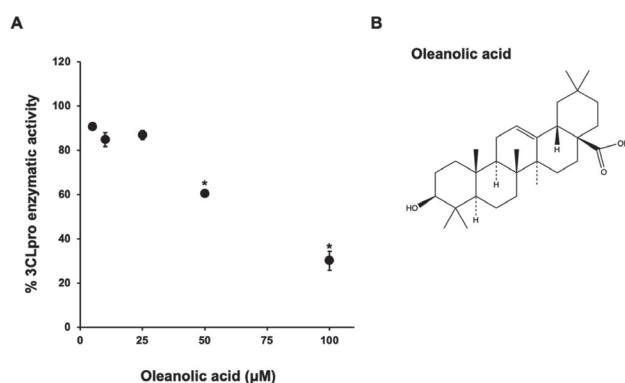
ue was subtracted from all other values.

**Statistical Analysis** Multiple comparisons were examined using ANOVA, followed by Dunnett's test.  $P < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

The fluorescence emitted when the substrate is cleaved by SARS-CoV-2 3CL<sup>pro</sup> is an excitation light at 360 nm and an emission light at 460 nm.<sup>7)</sup> Thus, we measured all phytochemicals at excitation and emission wavelengths of 360 and 460 nm, respectively, and no autofluorescence was observed. We then examined the inhibitory effect of phytochemicals on the activity of 3CL<sup>pro</sup>. Among these phytochemicals, 3-hydroxytyrosol, luteolin, oleanolic acid, oleocanthal, and verbascoside significantly inhibited the 3CL<sup>pro</sup> activity. Oleocanthal, 3-hydroxytyrosol, and verbascoside weakly inhibited the 3CL<sup>pro</sup> activity (~20%). However, luteolin and oleanolic acid inhibited the 3CL<sup>pro</sup> activity by about 40% (Fig. 1). GC376, which had been developed as an inhibitor of feline coronavirus and inhibits SARS-CoV-2 3CL<sup>pro</sup> activity,<sup>8,9)</sup> was used as a positive control for protease inhibition. Notably, phytochemicals, including luteolin and oleanolic acid, bind to 3CL<sup>pro</sup> via *in silico* studies.<sup>6,10–13)</sup> In addition, luteolin has been reported to inhibit 3CL<sup>pro</sup>.<sup>10)</sup> However, the actual enzyme inhibitory action of oleanolic acid has not been examined. In addition, 50  $\mu\text{M}$  oleanolic acid was used, but the amount added to the reaction was 10  $\mu\text{L}$ , which is 500 pmol, or about 0.2  $\mu\text{g}$ . Oleanolic acid at 250 mg/kg/d has also been used in animal experiments on mice.<sup>14)</sup> Thus, we examined the dose response in the inhibition of 3CL<sup>pro</sup> by oleanolic acid (Fig. 2A). Oleanolic acid significantly inhibited the enzymatic activity of 3CL<sup>pro</sup> at concentrations above 50  $\mu\text{M}$ ; the oleanolic acid concentration required for 50% inhibition ( $\text{IC}_{50}$ ) of 3CL<sup>pro</sup> activity was 51.4  $\mu\text{M}$ .

SARS-CoV-2 infects cells by binding angiotensin-converting enzyme 2 as a viral receptor. After viral entry, the viral genome RNA is released and a huge protein is translated into the cell. This huge protein, which contains NSPs, is mainly cleaved by 3CL<sup>pro</sup>. NSPs, including RdRP, are released and activated. NSPs are involved in viral replication and the affection of host cells. RdRP is essential for copying SARS-CoV-2 genome RNA. Thus, inhibiting 3CL<sup>pro</sup> is crucial for preventing the replication of SARS-CoV-2.



**Fig. 2.** (A) Dose-Dependent Effects of Oleanolic Acid on 3CL<sup>pro</sup> Activity

3CL<sup>pro</sup> inhibitory activity observed in the DMSO-treated control was 100%. Values are expressed as means  $\pm$  SEM (n = 3).  $p < 0.05$  was considered statistically significant. (B) Structure of oleanolic acid.

Several phytochemicals show an inhibitory effect on virus activities, such as the entry of the virus into host cells and the inhibition of virus enzymes. Olives contain phytochemicals, including polyphenols and terpenoids. Oleanolic acid is a pentacyclic triterpenoid (Fig. 2B) and is abundant in olives. It has various pharmacological activities, such as hepatoprotection, antioxidation, anticancer, and antiinflammation.<sup>15,16)</sup> In addition, oleanolic acid derivatives inhibit HIV protease.<sup>17)</sup> In our results, oleanolic acid shows the inhibitory effects of SARS-CoV-2 3CL<sup>pro</sup> in a dose dependent manner. 3CL<sup>pro</sup> is a cysteine protease and the consensus amino acid sequence, which is cleaved by 3CL<sup>pro</sup>, is the Leu-Gln↓ (Ser, Ala, Gly) sequence (cleavage sites are indicated by ↓).<sup>18)</sup> 3CL<sup>pro</sup> possesses catalytic dyad comprising cysteine 145 and histidine 41 in its catalytic site. In addition, the active form of 3CL<sup>pro</sup> is considered a homodimer and domain III of 3CL<sup>pro</sup> is involved in its dimerization.<sup>19)</sup> Oleanolic acid can interact with arginine 131 and aspartic acid 289 by *in silico* analysis.<sup>12)</sup> Thus, oleanolic acid might not bind to or inhibit the catalytic site. However, arginine 131 and aspartic acid 289 are associated with a salt bridge that stabilizes domains II and III to form a dimer of 3CL<sup>pro</sup>.<sup>19)</sup> Therefore, oleanolic acid might inhibit 3CL<sup>pro</sup> activity by interfering with the dimerization of 3CL<sup>pro</sup>. However, 50  $\mu\text{M}$  oleanolic acid is 22.8  $\mu\text{g/mL}$ , and it has been reported that oral single administration of 30 mg of oleanolic acid reaches 598.2  $\pm$  176.7 ng/mL in human serum.<sup>20)</sup> Although the inhibitory effect of oleanolic acid on SARS-CoV-2 3CL<sup>pro</sup> may not appear by a single oral administration of 30 mg of oleanolic acid in human, changing the dose or frequency of oral administration may increase the concentration of oleanolic acid in human serum. Furthermore, PVP-modified liposomes enhance the plasma concentration of oleanolic acid in rats.<sup>21)</sup> In addition, inhalation of budesonide (800  $\mu\text{g}$  twice daily for 14 d) improves time to recovery and reduces deaths in patients with COVID-19.<sup>22)</sup> Thus, to achieve inhibitory effects of oleanolic acid on COVID-19, the site and method of administration of oleanolic acid should be examined.

In conclusion, we examined the inhibitory effect of phytochemicals from olives on SARS-CoV-2 3CL<sup>pro</sup> and found that oleanolic acid statistically significantly inhibited 3CL<sup>pro</sup> activity. Although the inhibitory mechanism of oleanolic acid against 3CL<sup>pro</sup> is not elucidated, it is presumed that oleanolic acid may bind to and inhibit a site different from the catalytic

ic site by *in silico* studies.<sup>12,19</sup> Combinations of drugs with different mechanisms of action may be more effective than drugs prescribed alone. Thus, oleanolic acid and its derivatives may be potent molecules that inhibit SARS-CoV-2 replication.

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**Conflict of interest** The authors declare no conflict of interest.

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