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Punicalagin and zinc (II) ions inhibit the activity of SARS-CoV-2 3CL-protease *in vitro*

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Abstract. – OBJECTIVE: Coronavirus 2019 (COVID-19) has now been declared as a worldwide pandemic. Currently, no drugs have been endorsed for its treatment; in this manner, a pressing need has been developed for any antiviral drugs that will treat COVID-19. Coronaviruses require the SARS-CoV-2 3CL-Protease (3CL-protease) for cleavage of its polyprotein to yield a single useful protein and assume a basic role in the disease progression. In this study, we demonstrated that punicalagin, the fundamental active element of pomegranate in addition to the combination of punicalagin with zinc (Zn) II, appear to show powerful inhibitory activity against SARS-CoV-2.

MATERIALS AND METHODS: The 3CL protease assay kit was used to quantify 3CL protease action. The tetrazolium dye, MTS, was used to evaluate cytotoxicity.

RESULTS: Punicalagin showed inhibitory action against the 3CL-protease in a dose-dependent manner, and IC50 was found to be 6.192 μg/ml for punicalagin. Punicalagin (10 μg/mL) demonstrated a significant inhibitory activity toward 3CL-protease activity ($p < 0.001$), yet when punicalagin is combined with zinc sulfate monohydrate (punicalagin/Zn-II) extremely strong 3CL-protease activity ($p < 0.001$) was obtained. The action of 3CL-protease with punicalagin/Zn-II was decreased by approximately 4.4-fold in contrast to only punicalagin (10 μg/mL). Likewise, we did not notice any significant cytotoxicity caused by punicalagin, Zn-II, or punicalagin/Zn-II.

CONCLUSIONS: We suggest that these compounds could be used as potential antiviral drugs against COVID-19.

Key Words:
Punicalagin, Zinc II, SARS-CoV-2 3CL-Protease, SARS-CoV-2, COVID-19.

Introduction

Currently, Coronavirus 2019 (COVID-19) is the main pandemic of the century since more than 95,000,000 people have been infected with this disease and more than 2,032,750 deaths have been reported as of January 2020[1]. However, vaccination remains the best preventive measure against COVID-19 infections. Considering all aspects, inoculation viability is diminished due to a high transformation rate of COVID-19 infections, thereby causing adjustments in immunization composition that make vaccine development difficult and time-consuming[2].

Cleavage of viral polyproteins by proteases is an important aspect of the existing pattern of coronavirus, and a few viral proteases that are encoded in the coronavirus RNA are needed for the development of the viral proteins[3]. The

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SARS-CoV-2 3CL-Protease (3CL-protease) is the only cysteine protease found in coronaviruses. It separates the COVID-19 polyprotein at eleven moderated sites, and it is essential for replication of the virus[4-6]. Considering its significance, the 3CL-protease is a target for antivirals, and few antiviral candidates were identified using a 3CL-protease test[7-9]. The 3CL-protease is one of the best drug targets among different corona viruses and functions to prevent virus replication. Because no similar enzyme in humans exists, these drugs tend to be non-toxic *vivo*[10]. Currently, synthetic compounds focusing on the CL-protease have been identified for inhibiting the replication of coronaviruses in *vivo*[10].

The most abundant polyphenol in pomegranate is punicalagin, which has been implicated as the bioactive constituent responsible for > 50% of the juice's potent antioxidant activity[11]. Punicalagin (MW 1084.7) and its major degradation product, ellagic acid, are thought to be the major bioactive phytochemicals present in pomegranate peel extract[12]. It has been shown that punicalagin targets and inactivates herpes simplex virus (HSV)-1 particles and can prevent binding, attachment, penetration, and cell-to-cell spread of protease inhibitors thus inhibiting viral glycoprotein interactions with cell surface glycosaminoglycans[13]. Punicalagin has also been reported to be effective in abrogating infection caused by human cytomegalovirus, hepatitis C virus, dengue virus, measles virus, and respiratory syncytial virus (HCMV, HCV, DENV, MV, and RSV, respectively), at micromolar (μM) concentrations and in a dose-dependent manner without significant cytotoxicity[14]; therefore, the impact of punicalagin was not examined as an expected virucide.

Zinc (Zn) has been shown to have direct antiviral properties (for example, against flu) and antiviral activity when Zn (II) is combined and co-administered with plants[15]. Zn can inhibit the protease activity and polymerase enzymatic processes in addition to physical processes, such as virus attachment, infection, and uncoating[15]. The use of a combination of drugs is necessary and effective for reducing the risk of drug-resistant mutations.

In the current study, we analyzed the potentiated antiviral activity of punicalagin, Zn (II), and punicalagin combined with Zn (II) particles using an assay in which the activity of 3CL-Protease was inhibited *in-vitro*.

Materials and Methods

Materials

Punicalagin (purity ≥ 95%) and Zn sulfate monohydrate were purchased from Sigma Aldrich (Saint Louis, MO, USA).

Assay Protocol Against the 3CL Protease

The measure of the punicalagin, Zn II, or a combination of punicalagin with the Zn II on the activity of the 3CL protease was assessed using an improved 3CL Protease Assay Kit (BPS Bioscience, #78042, San Diego, USA). The fluorescence was estimated using a Tecan microplate fluorimeter equipped for excitation and emission at 360 and 460 nm, respectively (Tecan Biotek, Winooski, VT) according to the manufacturer's instructions described in the kit[16,17].

Cytotoxicity

Test solutions were analyzed for cytotoxicity using the Cell Titer 96 Aqueous Kit (Promega, Southampton, UK). Cells were plated in a 96-well-plate with a known concentration of Vero cells in each well. Cells (2.5×10^3) for each well was used for each plate after Vero cells had reached 80% confluence in a T75 flask (Greiner Bio-One, Stonehouse, UK). The cells were then plated in Dulbecco's Modified Eagle's Medium (DMEM) and cultivated for 24 hours at 37°C under 5% CO_2.

The medium was removed by aspiration, and the cells were washed multiple times with phosphate-buffered saline (PBS) after which the PBS was quickly replaced with DMEM media containing the ideal concentrations of test materials. Cells were incubated for 6, 24, 48 and 72 h. Cell titers were used to identify cell viability and were added to each well. Plates were incubated for 1 h at 37 °C under 5% CO_2. Optical thickness was then measured at 492 nm with the blank value subtracted from each sample reading and the mean thickness for the control cells were assigned a value of 100%.

Statistical Analysis

Data analysis was performed with the GraphPad Prism package and SPSS software. Differences among the studied groups were determined based on one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons as post-hoc test. $p < 0.05$ was considered significant.

Results

Inhibitory Effect of Punicalagin on 3CL Protease Activity

The activity of the 3CL-protease was determined (Figure 1). Likewise, we inspected the impact that punicalagin had on the 3CL-protease activity by testing different concentrations of punicalagin (0, 1, 2, 5, 10, 20, and 40 μg/ml). We found that punicalagin inhibited 3CL-protease activity (Figure 1). The half maximal inhibitory concentration (IC_{50}) of punicalagin was 6.192 μg/mL (Figure 2). These results show that punicalagin is an inhibitor of 3CL-protease. Increasing the concentration of punicalagin above 10 μM did not yield any further inhibition of the protease (Figure 2). Thus, in resulting tests, 10 μm punicalagin was used.

The Inhibitory Effect of Punicalagin When Combined with Different Levels of Zinc Sulfate Monohydrate (Punicalagin/Zn-II) on 3CL Protease Activity

The impact of 10 μg/ml punicalagin containing different concentrations of Zn sulfate monohydrate (0, 0.2, 0.5, 3, 5, 30, and 100 mg/ml) on the activity of the 3CL-protease was evaluated. The outcomes indicated that Zn sulfate inhibited 3CL-protease activity (Figure 3). These outcomes demonstrate that punicalagin is a more powerful inhibitor of 3CL-protease action than Zn sulfate.

Increasing levels of Zn sulfate were associated with a decrease in the activity of 3CL protease.

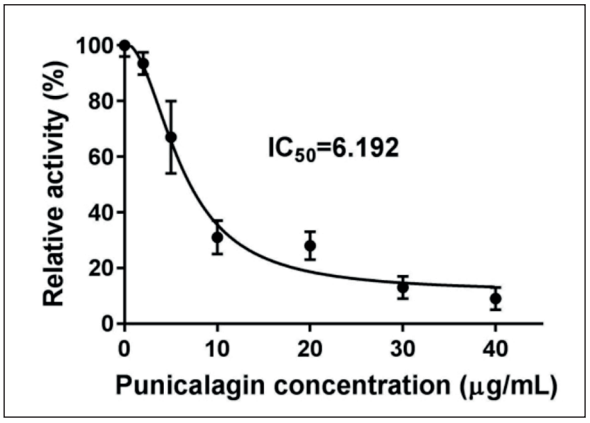

Figure 2. The half maximal inhibitory concentration (IC_{50}) of punicalagin.

The maximum reduction in activity was observed when a combination of punicalagin with 3 mg/ml Zn sulfate monohydrate (Figure 3) was used. Increasing the concentration of Zn sulfate monohydrate to > 3 mg/mL did not cause an increase in its inhibitory effects.

Both punicalagin and Zn sulfate inhibited the 3CL protease. We analyzed whether punicalagin and Zn sulfate monohydrate together may have had an added or a synergistic inhibitory impact on 3CL-protease activity. We incubated cells with punicalagin alone, Zn sulfate alone, and punicalagin/Zn sulfate combination and estimated the inhibitory impact on the 3CL-protease. Using the observed protease activity, we determined the coefficient of drug association (CDI) and determined a CDI for punicalagin (10 μg/mL)

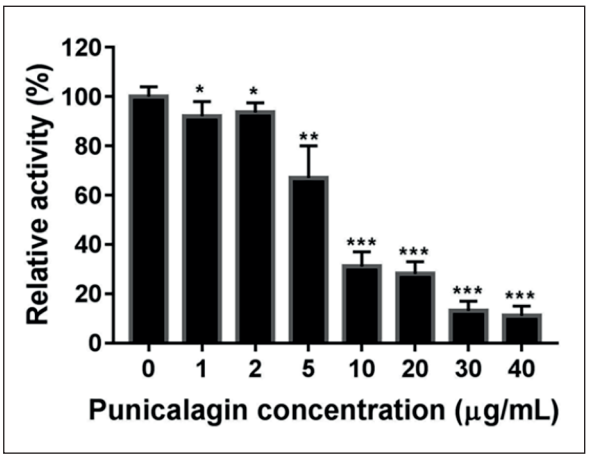

Figure 1. Punicalagin inhibits 3CL-protease *in vitro* at different concentration. The 3CL-protease activity was analyzed in triplicate, and the mean and standard deviation are shown. *p>0.05, **p<0.01, ***p<0.001.

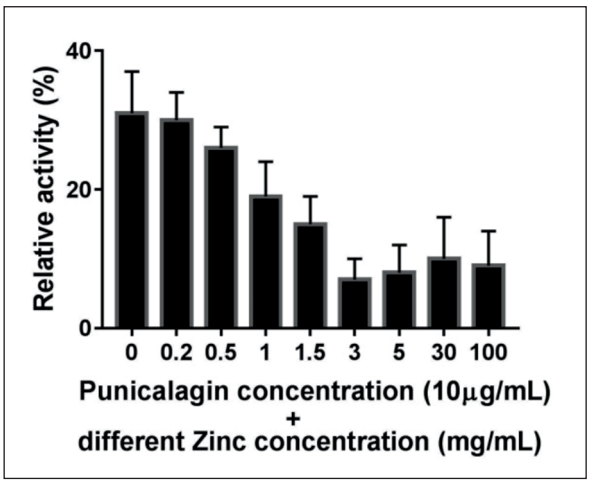

Figure 3. Punicalagin (10μg/ml) with different zinc (Zn) sulfate monohydrate concentration inhibits 3CL-protease *in vitro*. The 3CL-protease activity was performed in triplicate.

and Zn sulfate monohydrate (3 mg/mL) of 0.272, demonstrating that punicalagin and Zn sulfate had a synergistic effect on the protease's activity (Figure 4).

For punicalagin/Zn-II, a 24% (4.4-fold) decrease in protease activity was estimated when compared with punicalagin alone (10 μg/ml) as shown in Figure 5.

Cytotoxicity

The cytotoxicity of 10 μg/mL punicalagin, 3 mg/mL Zn sulfate monohydrate, and combination of 10 μg/mL punicalagin with 3 mg/mL Zn sulfate monohydrate were evaluated using the tetrazolium dye, MTS, proliferation assay. No significant differences ($p > 0.05$) between the applied formulations any time over the 72 h were observed, indicating these drugs did not affect cell viability (Figure 6).

Discussion

The need for a COVID-19 treatment is critical as COVID-19 is spreading quickly, and infections/deaths are constantly rising worldwide. The 3CL protease is a promising target for COVID-19 treatment. Recently, a peptidomimetic α-ketoamide compound was demonstrated to inhibit the 3CL-protease and decrease viral replication in cell culture[10]. Likewise, epigallocatechin-3-gallate (EGCG) and theaflavin appear to be promising compounds for inhibiting the 3CL-protease *in-vitro*[11,18]. Nonetheless, further stringent testing should be done place to determine the safety and viability of these chemicals.

In this investigation, we analyzed whether punicalagin and punicalagin/Zn-II, the significant active ingredient of pomegranates, have inhibitory activity against the 3CL-protease. We showed that both punicalagin and punicalagin/Zn-II inhibited 3CL-protease activity. We found that an IC_{50} of 6.192 μg/mL for punicalagin was the critical concentration for causing a decrease in 3CL protease activity ($p < 0.001$) at 10 μg/mL of punicalagin. The constituents of pomegranate peel extract, to be specific punicalagin and punicalin, have the promising potential for critical

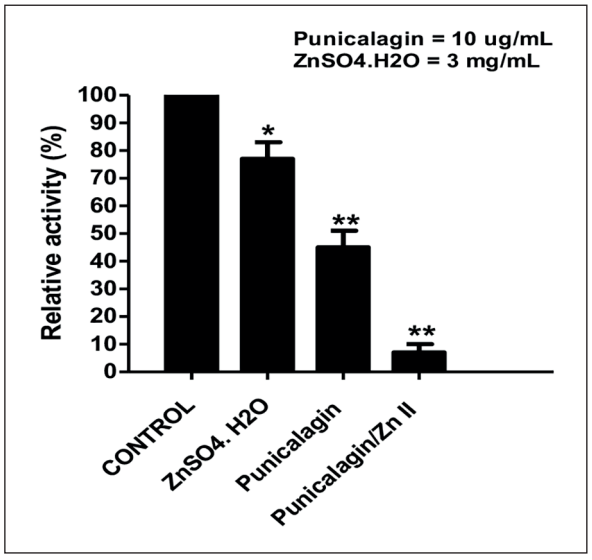

Figure 4. The activity assay of Zn sulfate monohydrate, Punicalagin, and Punicalagin/Zn-II against 3CL-protease activity; *p<0.01, **p<0.001.

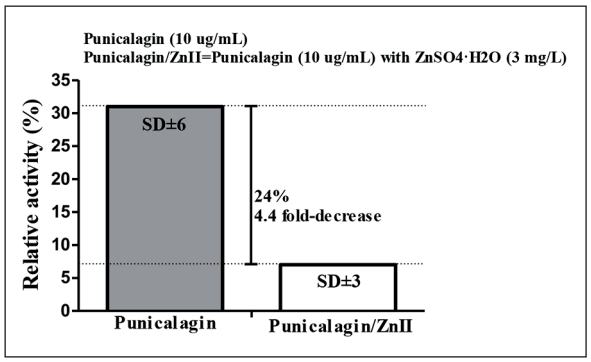

Figure 5. The percentage decrease activity and fold decrease activity assay for both Punicalagin, and Punicalagin/Zn-II against 3CL-protease activity.

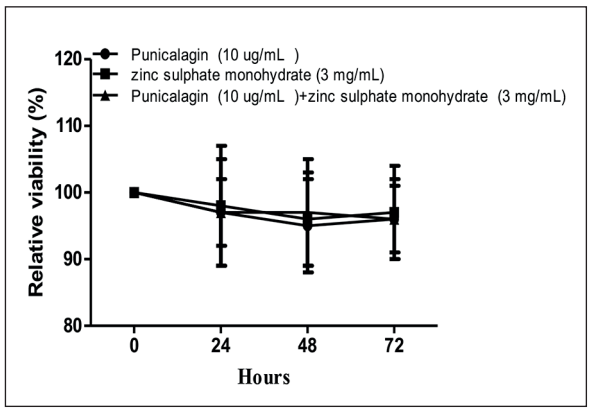

Figure 6. Cytotoxicity Effect of (1) Punicalagin (10 μg/mL), (2) zinc sulphate monohydrate (3 mg/mL), (3) the combination of Punicalagin (60μM) + zinc sulphate monohydrate (3 mg/mL). No cytotoxic effects were observed for all of the compounds at the concentrations used in this study.

interactions with the chosen protein targets and are accordingly considered acceptable candidates for additional *in vitro* and *in vivo* assessments[19]. A similar study showed that both theaflavin and EGCG produced a dose-dependent inhibitory effect on the 3CL protease. The IC_{50} values were was 8.44 μg/ml and 7.58 μg/ml for theaflavin and EGCG, respectively[19].

Moreover, ZnII had direct antiviral properties against infections since it can impede protease activity and polymerase enzymatic cycles[20]. Zn particles have recently been demonstrated as strong inhibitors of different RNA infections[21]. We observed that Zn induced a significant decline in 3CL-protease activity ($p < 0.01$).

One of the main questions about these compounds is whether their mixture increments could lead to 3CL-protease inhibition. Using a combination of 10 μg/mL punicalagin and 3 mg/mL Zn sulfate monohydrate, we noticed a significant decrease in 3CL-protease activity ($p < 0.001$), while not causing recognizable cytotoxicity. Inhibition of replication induced by pyrithione and Zn^{2+} over a range from 2 to 10 μM was recently reported for a few picornaviruses, for example, rhinoviruses, foot-and-mouth infections, coxsackievirus, and mengovirus[22,23].

Targeting the SARS enzyme as a potential target for drug discovery was done because of the lack of homologues in human hosts. Inhibitors of this enzyme have the capability of reducing viral replication and transcription without causing cell toxicity. In coronaviruses, Zn particles seem to inhibit both the proteolytic handling of replicate polyproteins[24] and SARS-CoV RdRp activity[21]. Likewise, late computational investigations have confirmed that polyphenols, for example, punicalagin, are inhibitors of SARS-CoV-2[19].

Conclusions

Summarily, the combination of punicalagin and Zn particles inhibit the SARS-CoV-2 3CL-protease *in vitro*. In this study, we demonstrated that punicalagin, the active ingredient of pomegranate, is a powerful to inhibitor of the 3CL protease *in-vitro*. In this way, pomegranate would be important for analyzing the impact of its active compound on the spread of SARS-CoV-2 *in vivo*. Also, further preliminary clinical trials will be needed to evaluate the impact of pomegranate use on the spread of COVID-19.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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