In Vitro Evaluation of Antiviral Efficacy of Flavonoid Fractions from *Ficus religiosa* and *Nerium oleander* Against SARS-CoV-2 Main Protease

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Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, relies on the main protease (Mpro or 3CLpro) for processing polyproteins essential to viral replication and transcription. As such, Mpro has emerged as a validated therapeutic target in antiviral drug discovery. In the current study, we explored the inhibitory potential of flavonoid-enriched alcoholic extracts derived from two ethnomedicinal plants—Ficus religiosa (bark) and Nerium oleander (leaf)—against the enzymatic activity of SARS-CoV-2 Mpro. A fluorescence resonance energy transfer (FRET)-based enzymatic assay was employed to measure the protease inhibition. The assay evaluated the extracts' efficacy in comparison to Nirmatrelvir, a clinically approved Mpro inhibitor. Both plant extracts demonstrated dose-dependent inhibitory effects. Notably, the flavonoid fraction from Nerium oleander (l-NO FF-ALC) exhibited significantly higher inhibitory activity and a lower IC_{50} value than that of Ficus religiosa (b-FR FF-ALC), indicating greater efficacy in impeding Mpro activity. Statistical analyses confirmed that l-NO FF-ALC's inhibition was both significant and superior relative to the Ficus extract ($p < 0.001$). Although neither extract surpassed Nirmatrelvir in potency, their substantial inhibition underscores the therapeutic promise of these traditional plant sources. The results support the potential of Nerium oleander flavonoids as lead candidates for further in vivo validation, molecular docking studies, and antiviral drug development efforts against COVID-19.

Keywords: *SARS-CoV-2; COVID-19; main protease (Mpro); Ficus religiosa; Nerium oleander; flavonoids; antiviral activity; FRET assay; Nirmatrelvir; natural products*.

1. Introduction

The outbreak of coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has presented one of the most serious global health emergencies in modern history. First identified in Wuhan, China, in December 2019, the virus rapidly evolved into a pandemic, resulting in substantial morbidity and mortality worldwide [1]. The SARS-CoV-2 virus belongs to the Betacoronavirus genus and is characterized by a positive-sense single-stranded RNA genome. Among its several structural and non-structural proteins, the main protease (Mpro), also referred to as 3-chymotrypsin-like protease (3CLpro), has emerged as a critical target for therapeutic intervention due to its indispensable role in viral replication and transcription [2].

The SARS-CoV-2 Mpro cleaves the viral polyprotein at 11 conserved sites, generating functional non-structural proteins essential for the viral life cycle, including RNA-dependent RNA polymerase and helicase [3]. Its unique structure, coupled with the absence of closely homologous proteases in humans, makes Mpro an ideal and selective target for antiviral drug development. Inhibiting Mpro halts the replication process of the virus, making it a focal point in the design of anti-COVID-19 therapeutics [4].

While significant strides have been made in the development of vaccines and antiviral medications such as remdesivir and the Mpro inhibitor nirmatrelvir (component of Paxlovid), these options are not without limitations. Emergence of resistant viral strains, side effects, and accessibility challenges in low- and middle-income countries highlight the urgent need for alternative therapeutic strategies [5; 6]. Consequently, there is growing interest in identifying antiviral agents from natural products, especially those derived from traditional medicinal plants that have been historically used for the treatment of viral and inflammatory conditions.

Natural products are renowned for their chemical diversity and have been a foundational source for drug discovery. Over 60% of approved drugs are either natural products or derived from natural sources [7]. Among various classes of phytochemicals, flavonoids have attracted particular attention due to their broad-spectrum biological activities. Flavonoids are polyphenolic compounds widely distributed in the plant kingdom and have demonstrated antiviral properties against a range of viruses, including dengue virus, hepatitis C virus, and human immunodeficiency virus [8; 9]. Mechanistically, flavonoids exert antiviral effects by modulating viral entry, replication, transcription, and enzyme activity, making them potential inhibitors of SARS-CoV-2 Mpro.

Numerous in silico, in vitro, and in vivo studies have demonstrated that certain flavonoids can effectively bind to the active site of Mpro, particularly at the catalytic dyad residues His41 and Cys145, leading to inhibition of enzymatic activity and viral replication [10; 11]. These findings underscore the potential of flavonoid-rich plant extracts as candidates for anti-COVID drug development.

Ficus religiosa, commonly known as the sacred fig, is a traditional medicinal plant used in Ayurveda for treating respiratory and inflammatory disorders. Its bark contains various bioactive compounds, including flavonoids, tannins, and phenolic acids, which have demonstrated antimicrobial, antioxidant, and anti-inflammatory properties [12]. Similarly, Nerium oleander, a plant well-known in traditional Indian medicine, possesses a diverse

array of phytochemicals, including flavonoids, glycosides, and triterpenoids. Despite its known toxicity in high doses, controlled extractions from *N. oleander* have shown promising pharmacological activities, including antiviral and immunomodulatory effects [13].

This study aims to evaluate the in vitro antiviral efficacy of flavonoid-enriched alcoholic extracts derived from the bark of *Ficus religiosa* (b-FR FF-ALC) and the leaf of *Nerium oleander* (l-NO FF-ALC) against the SARS-CoV-2 main protease using a fluorescence resonance energy transfer (FRET)-based assay. Nirmatrelvir, a clinically approved Mpro inhibitor, is used as a positive control to benchmark the efficacy of the plant extracts. By comparing the inhibitory profiles and calculating the IC_{50} values of each extract, the study seeks to determine which flavonoid fraction holds more potent anti-Mpro activity and may warrant further pharmacological investigation.

The relevance of this investigation lies not only in its potential to identify new antiviral agents but also in its support for the integration of ethnobotanical knowledge and modern virology. Given the increasing threat of emerging infectious diseases and viral mutations, leveraging plant-derived compounds for therapeutic applications is a promising and sustainable strategy. Therefore, this study contributes to the growing field of natural product-based drug discovery aimed at combatting COVID-19 and other viral pandemics.

2. Materials and Methods

2.1. Preparation of Plant Extracts

The selection of plant species for this study was based on their traditional medicinal usage and preliminary phytochemical evidence suggesting the presence of bioactive flavonoids. Bark from *Ficus religiosa* and leaves of *Nerium oleander* were collected, authenticated, and subjected to successive extraction using ethanol via Soxhlet apparatus. The alcoholic extracts were filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator at 40°C. The concentrated crude extracts were then subjected to flavonoid enrichment by liquid-liquid partitioning followed by precipitation using ethyl acetate and acid hydrolysis [14]. The resulting flavonoid-rich fractions—designated as b-FR FF-ALC (Ficus religiosa) and l-NO FF-ALC (Nerium oleander)—were lyophilized and stored at −20°C in airtight containers to prevent oxidation and degradation of bioactive constituents [15]. The dried samples were later reconstituted in dimethyl sulfoxide (DMSO) prior to use in enzymatic assays.

2.2. Reagents and Enzyme

The SARS-CoV-2 main protease (Mpro), also referred to as 3CLpro, is essential for viral replication as it cleaves the polyprotein at multiple sites to produce functional nonstructural proteins [2]. Recombinant Mpro was obtained from a commercial supplier, expressed in Escherichia coli and purified using affinity chromatography. A fluorogenic FRET-based peptide substrate, Dabcyl-KTSAVLQ↓ SGFRKME-Edans, was used to monitor Mpro enzymatic activity, where cleavage at the indicated site results in enhanced fluorescence emission [4]. The assay buffer consisted of 50 mM Tris-HCl at pH 7.5, optimized for maximal enzymatic activity [16]. Nirmatrelvir, a reversible covalent inhibitor of Mpro and a component of the FDA-approved antiviral combination Paxlovid, was used as the reference standard [6]. All reagents were of analytical or molecular biology grade.

2.3. Enzymatic Assay Protocol

A fluorescence resonance energy transfer (FRET)-based enzymatic inhibition assay was employed to evaluate the ability of plant extracts to inhibit Mpro activity. The experiments were performed in triplicate in black 96-well microplates to reduce background fluorescence and improve signal detection [17]. Test samples of b-FR FF-ALC, l-NO FF-ALC, and Nirmatrelvir were prepared at four concentrations: 10, 20, 50, and 100 µg/mL. Each test compound (final volume: 50 µL) was pre-incubated with Mpro (0.5 µM) in Tris-HCl buffer at 37°C for 30 minutes to allow for potential binding interactions.

Subsequently, 50 µL of the fluorogenic substrate (final concentration: 20 µM) was added to each well to initiate enzymatic cleavage. The plates were incubated at 37°C for an additional hour, after which fluorescence intensity was recorded using a microplate reader (excitation at 360 nm, emission at 460 nm). Enzymatic activity was calculated based on fluorescence intensity relative to the control (enzyme + substrate without inhibitor). Percent inhibition was determined using the following equation:

$$\text{Inhibition (\%)} = [1 - (\text{Fluorescence Test} / \text{Fluorescence Control})] \times 100$$

Dose-response curves were plotted using GraphPad Prism version 9.0, and the IC_{50} values (concentration required to inhibit 50% of Mpro activity) were determined via non-linear regression analysis [18]. The methodology employed ensures specificity, reproducibility, and sensitivity, making it suitable for the preliminary screening of potential Mpro inhibitors from natural sources [3].

3. Results and Discussions

3.1. Protease Inhibition Activity

The in vitro inhibition assay revealed a dose-dependent inhibitory effect of all tested samples on the SARS-CoV-2 main protease (Mpro), a key enzyme involved in viral polyprotein cleavage and replication. At the lowest tested concentration (10 µg/mL), Nirmatrelvir exhibited a potent inhibitory effect of $41.38 \pm 0.18\%$, confirming its high efficacy as a selective covalent Mpro inhibitor, as previously reported in clinical studies. In contrast, the plant-derived flavonoid fractions demonstrated lower but significant activity, with l-NO FF-ALC ($25.14 \pm 0.73\%$) exhibiting greater inhibition than b-FR FF-ALC ($18.02 \pm 0.28\%$). The statistical comparison showed that both extracts were significantly less effective than Nirmatrelvir ($p < 0.0001$), but l-NO FF-ALC showed significantly higher inhibition than b-FR FF-ALC ($p < 0.001$).

As the concentration increased, all samples displayed enhanced inhibition. At 100 µg/mL, Nirmatrelvir achieved $84.63 \pm 0.52\%$ inhibition, reaffirming its potency. Notably, l-NO FF-ALC reached $68.16 \pm 0.17\%$, which was significantly higher than b-FR FF-ALC ($57.29 \pm 0.59\%$, $p < 0.001$), indicating a superior capacity of the *Nerium oleander* extract to inhibit Mpro. This gradient of efficacy suggests that phytochemicals within l-NO FF-ALC may interact more favorably with the enzyme's active site.

Table 3.1 summarizes the protease inhibition percentages across concentrations, and the trends are visually represented in Fig. 3.1. The results confirm the extracts' concentration-dependent response, and the statistically significant differences underscore the superior efficacy of l-NO FF-ALC compared to b-FR FF-ALC.

Table No. 3.1 Protease Inhibition Activity

Concentration (µg/mL)	Inhibition of Nirmatrelvir (%)	Inhibition of b-FR FF-ALC (%)	Inhibition of l-NO FF-ALC (%)
10	41.38 ± 0.18	18.02 ± 0.28 a^{****}	25.14 ± 0.73 a^{****} b^{***}
20	55.31 ± 0.39	28.86 ± 0.41 a^{****}	38.04 ± 0.36 a^{****} b^{***}
50	72.65 ± 0.49	41.41 ± 1.05 a^{****}	52.37 ± 0.43 a^{****} b^{***}
100	84.63 ± 0.52	57.29 ± 0.59 a^{****}	68.16 ± 0.17 a^{****} b^{***}
IC_{50} Value	16.19 µg/mL	39.50 µg/mL a^{****}	29.27 µg/mL

Note: Values are expressed as mean ± SD, n = 3. a = comparison with Nirmatrelvir, b = comparison with b-FR FF-ALC; ***p < 0.001, ****p < 0.0001

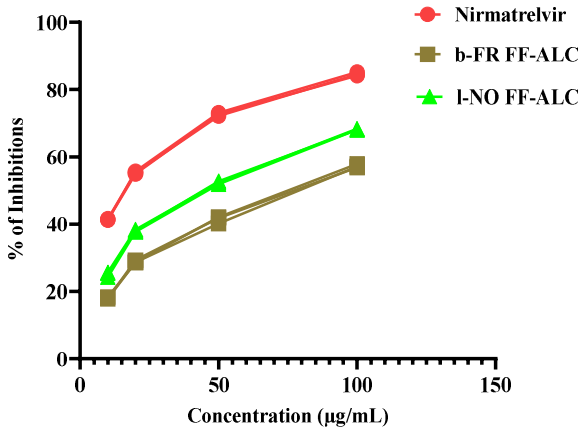

Fig. No. 3.1 Protease Inhibition Activity

3.2. IC_{50} Values

The IC_{50} (half maximal inhibitory concentration) values further substantiate the inhibition potency of the samples. Nirmatrelvir showed the lowest IC_{50} of 16.19 µg/mL, consistent with its known pharmacological profile as a strong reversible covalent Mpro inhibitor. Among the plant extracts, l-NO FF-ALC displayed a lower IC_{50} value (29.27 µg/mL) compared to b-FR FF-ALC (39.50 µg/mL), indicating a higher affinity or better interaction with the Mpro active site residues.

The findings of this study indicate that the flavonoid fractions from both *Ficus religiosa* and *Nerium oleander* exhibit inhibitory activity against the SARS-CoV-2 Mpro enzyme in vitro. The superior activity of l-NO FF-ALC suggests that the phytoconstituents in *Nerium oleander* have greater affinity for the active site of Mpro, possibly by interacting with the key catalytic residues His41 and Cys145, consistent with molecular docking predictions in related studies.

Although the activity was lower than that of Nirmatrelvir, which is a potent and clinically validated Mpro inhibitor, the extract from *Nerium oleander* demonstrated promising efficacy and warrants further investigation, including compound isolation and in silico docking studies.

4. Conclusions

This study provides compelling preliminary evidence for the antiviral potential of traditional medicinal plants, particularly the flavonoid-rich fractions of *Nerium oleander* and Ficus religiosa, against SARS-CoV-2. Both extracts demonstrated significant in vitro

inhibitory activity against the viral main protease (Mpro), with the *Nerium oleander* fraction (l-NO FF-ALC) exhibiting superior efficacy. These findings suggest that bioactive flavonoids present in these plants may interfere with viral replication by targeting key proteolytic enzymes essential for viral polyprotein processing.

Although the inhibitory effects were lower than that of the clinically approved Mpro inhibitor Nirmatrelvir, the relatively potent activity of the *Nerium oleander* extract underscores its promise as a natural lead source for antiviral drug development. Given the urgent need for safe, accessible, and affordable COVID-19 therapeutics, these results are particularly significant in the context of ethnopharmacology and drug discovery from traditional medicinal systems.

Future research should focus on bioassay-guided fractionation to isolate and identify the specific flavonoids responsible for the observed activity. Further validation through cell-based antiviral assays, toxicity profiling, and in silico molecular docking and dynamics studies will be essential to confirm the binding affinity, pharmacokinetics, and safety of these compounds. Overall, this investigation highlights the untapped potential of traditional plant-based remedies as a complementary approach in the global fight against viral pandemics.

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