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In Vitro Inhibitory Effect of *Nigella sativa* L. Extracts on SARS-COV-2 Spike Protein-ACE2 Interaction

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ABSTRACT

Ethnopharmacological relevance: A catastrophic outbreak of severe acute respiratory syndrome (SARS-CoV-2; Coronavirus disease-19; COVID-19) was first detected in Wuhan, Hubei Province, China, in December 2019. Many experimental and clinical studies have focused on the effectiveness of medicinal plants such as *Nigella sativa* (NS) in combating SARS-CoV-2. In this study, we aimed to evaluate the *in vitro* effect of NS seed extract on SARS-CoV-2 spike protein (S1)-angiotensin-converting enzyme 2 (S-ACE2) interaction.
Materials and methods: NS seed extracts used for the assay were prepared in chloroform, ethanol, and water by Soxhlet extraction and recovered by rotary evaporation. The inhibition percentage of S1-ACE2 interaction was analyzed using ELISA-SARS-CoV-2 S1 Protein-ACE2 Binding Inhibitor Screening Kit. Chemical finger-printing of the extracts was done using RP-HPLC.
Results: Significant concentration-dependent inhibition of the S1-ACE2 interaction was observed with chloroform, ethanol, and water extracts, ranging from 0.01 to 10 mg/ml. The *P*-values for the extracts were as follows: 0.0055, 0.0937, 0.0013, and 0.0003 for chloroform extract; 0.0876, 0.0703, 0.0183, and 0.0071 for ethanol extract; and 0.0915, 0.0312, 0.0006, and 0.0006 for water extract. The 50% inhibitory concentration (IC50) was determined to be 0.132 mg/ml, 0.288 mg/ml, and 4.06 mg/ml for chloroform, ethanol, and water extracts, respectively.
Conclusion: The *in vitro* analysis utilizing SARS-CoV2 spike (S1) and ACE2 proteins proved that NS seed extracts have the potential to inhibit the S-ACE2 interaction, which warrants further studies that could lead to potential drug discovery for SARS-CoV2 infection.

Introduction

A global outbreak of SARS-CoV-2 virus caused 6,974,473 deaths worldwide that led World Health Organization (WHO) to declare the outbreak as a Public Health Emergency of International Concern (PHEIC), defining it as a pandemic on 11 March 2020. The COVID-19 symptoms range from minor fever, cough, and shortness of breath to severe clinical conditions marked by respiratory failure and other prolonged medical disorders.[1] SARS-CoV-2, which belongs to the betacoronavirus genus, anchors onto the host cell with the help of the spike (S) glycoprotein on its envelope, which is then cleaved by furin into S1 and S2 subunits. The receptor binding domain (RBD) of the S1 subunit binds to the external surface of the claw-like structure of the host cell receptor, angiotensin-converting enzyme 2 (ACE2), and the S2 subunit aids in virus fusion onto the host cell membrane.[2] This makes ACE2 one of the potential targets for antiviral intervention against COVID-19.[3] A wide range of herbs and natural compounds have been reported for their promising therapeutic ability against SARS-CoV2.[4] Alternative therapies based on traditional medicines or natural products have been a promising strategy to prevent infection or to be used as adjunct ther-

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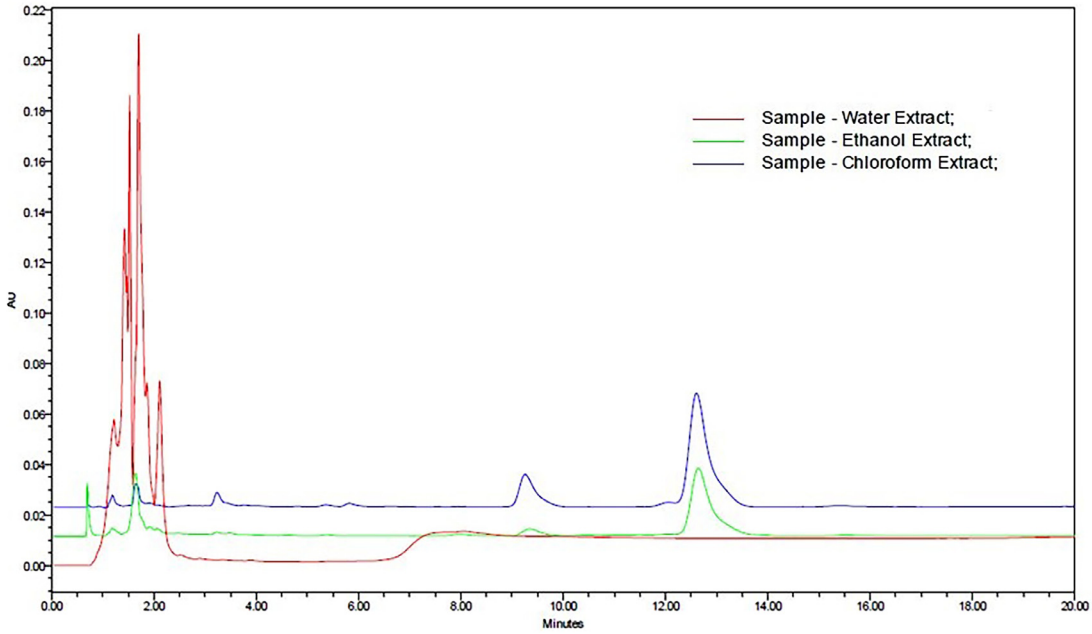

Figure 1. Reverse phase—HPLC of NS extract.

apy to mainstream treatment to reduce illness severity.[5] *Nigella sativa* (NS) is a promising herb with a wide pharmacological spectrum that has been used for centuries for its antiviral and immunomodulatory activities.[6] Numerous molecular docking studies of NS compounds such as thymoquinone (TQ), dithymoquinone, nigellidine, alpha-hederin, alpha-thujene, m-cymene, D-Limonene, D-terpinene, terpineol, and bisabolene reported a favorable role in S-ACE2 interaction inhibition.[6–9] In addition to *in silico* studies, many clinical trials and *in vitro* studies have shown that NS, is effective against COVID-19 patients.[10–13] The aim of this work focused on the evaluation of in *vitro* ACE2 inhibitory impact of NS seed extracts against S1 subunit of the spike protein. The findings from this study suggest that the compounds present in NS may be effective candidates for inhibition of S1-ACE2 interaction and warrant further studies to determine its potential clinical use.

Materials and methods

Plant materials and preparation of seed extracts

NS commonly known as black seed or kalonji, purchased from the local market in Dubai (UAE), were authenticated based on morphology and histology at the Department of Clinical Pharmacy and Pharmacotherapeutics, Dubai Pharmacy College, UAE. A sample of the seeds has been kept at Mohammed Bin Rashid University, Dubai, UAE, with reference number: MBRU230900. For the extraction, air-dried plant powdered seeds of NS were used. Using the Soxhlet equipment, extracts were made from 20 g of powder in 200 ml of solvents of different polarities (chloroform, ethanol, and water) followed by evaporation of solvents by a rotary evaporator (BUCHI rotavapor) at 50°C under reduced pressure and refrigerated until use. The percentage of extract yield was calculated using the formula, Y(yield) = (We*100)/Wt, where We and Wt denote the weight of completely dried extract and the weight of ground NS seeds prior to extraction respectively.

HPLC characterization

Reversed-phase high-performance liquid chromatography (RP-HPLC) technique was utilized to generate a chemical fingerprint of the NS seed extracts. The RP-HPLC analysis of the NS extracts was carried out with a Waters HPLC system (USA), equipped with a binary pump, auto-sampler, UV-Visible detector, and Breeze-2 software. The sample was prepared using Mili Q Ultrapure (Type 1) water (Millipore, Bedford, MA, USA) for water extracts and methanol for ethanol/chloroform extracts, sonicated for 15 minutes and filtered using 0.45 µM membrane filter. The sample was run with a C-18 column (Pinnacle DB, 5 µm, 4.6 × 150 mm, Restek, USA) with the mobile phase composition of methanol: water (1:1, v/v) at 254 nm with a flow rate of 1 mL/min. The injection volume was 10 uL and the sample was run for 20 minutes with an isocratic mode.

ELISA

The S1-ACE2 interaction inhibition assay was performed according to the manufacturer's instructions using a commercially available SARS-CoV-2 S1 Protein-ACE2 binding inhibitor screening ELISA kit (Abcam, ab283370). Before the assay, a stock solution of the extracts was prepared in dimethylsulfoxide (DMSO) (100 mg/mL). The extracts were then tested at concentrations ranging from 0.01 to 10 mg/mL. To prepare the required concentrations, serial dilution of the stock solution was performed using the sample diluent provided in the kit. The assay was carried out in duplicates along with the positive control provided with the kit. The inhibition percentage for each NS concentration was calculated at 650 nm and 450 nm using Hidex reader according to the formula mentioned below:

$$Relative\ inhibition\ (\%) = \frac{OD\ (Binding\ control) - OD\ (Sample\ compound) x100}{OD\ (Binding\ control)}$$

OD - Optical density

Statistical analysis

Data are expressed as mean ± SEM. Dose-response curve, IC50 and statistical analysis using one-way analysis of variance (ANOVA) were performed using GraphPad Prism version 9.4.1 for Windows

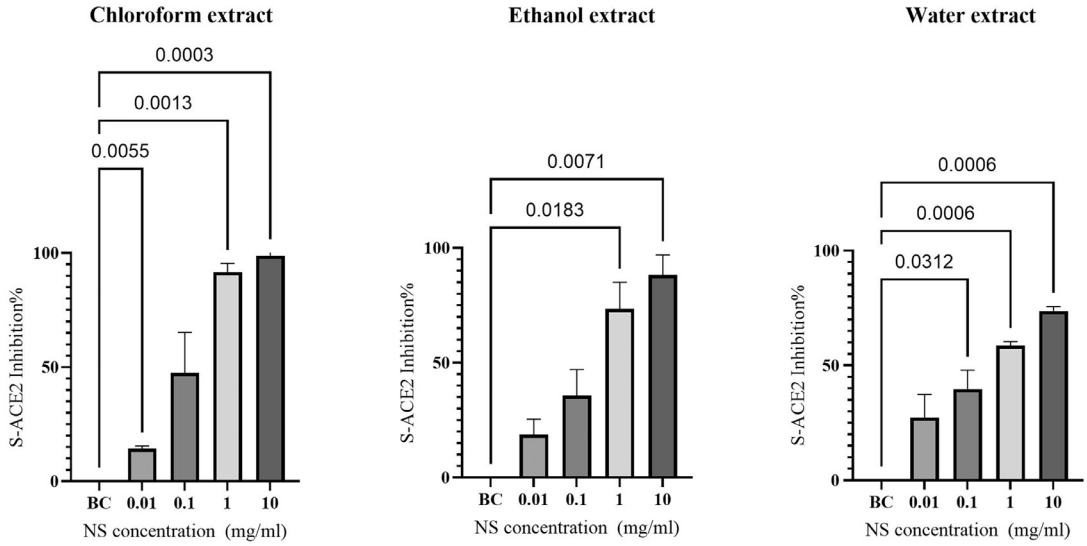

Figure 2. S1-ACE2 inhibition % for chlororform, ethanol, and water extracts of NS at concentrations ranging from 0.01 to 10 mg/ml.

Table 1
Concentration of NS extracts and average percentage inhibition for the triplicates (mean ± SEM) in different solvents.

NS concentration (mg/ml)	Chloroform extract	Ethanol extract	Water extract
10	**98.9 ± 1.1**	**88.3 ± 5.02**	**75.1 ± 1.19**
1	**91.65 ± 2.21**	**73.4 ± 6.73**	**60.4 ± 0.96**
0.1	**47.5 ± 10.18**	**35.66 ± 6.55**	**47.5 ± 4.76**
0.01	**14.3 ± 0.71**	**18.7 ± 3.86**	**38.2 ± 5.80**

(GraphPad Software, San Diego, USA). *P*-values ≤ .05 were considered statistically significant.

Results

Serial dilutions of the NS extracts were used in the ELISA to study the S1-ACE2 interaction, and the percentage inhibition was calculated from the measured absorbance values. The extract yield percentages for chloroform, ethanol, and water were 36%, 16%, and 4%, respectively. RP-HPLC analysis confirmed the presence of phytoconstituents present in the extracts (Figure 1). As the extract concentrations were increased from 0.01 to 10 mg/mL, the percentage inhibition in chloroform, ethanol and water extracts increased from 14.3% to 98.9%, 18.7% to 88.3%, and 38.2% to 75.1%, respectively (Table 1; Figure 2). For concentrations ranging from 0.01 to 10 mg/ml, NS extracts inhibited S1-ACE2 interaction with significant *P* values of 0.0055, 0.0013, and 0.0003 for chloroform extract; 0.0183 and 0.0071 for ethanol extract; 0.0312, 0.0006, and 0.0006 for water extract.

The IC50 of 0.132 mg/ml, 0.288 mg/ml, and 4.06 mg/ml for chloroform, ethanol, and water extracts, respectively, were derived from the dose-response curve using GraphPad Prism as illustrated in Figure 3. Inhibition % were also compared between all three extracts and the resulting *P* values are illustrated in Figure 4. The effectiveness of chloroform extract could be due its lipophilic and nonpolar organic nature which elutes out nonpolar compounds such as fats, volatile oils, terpenoids, saponins, tannins, and flavonoids, yielding extracts enriched with these active phytochemical constituents. Water and ethanol being polar in nature is used to extract out highly polar molecules such as organic acids and alkaloids.[14]

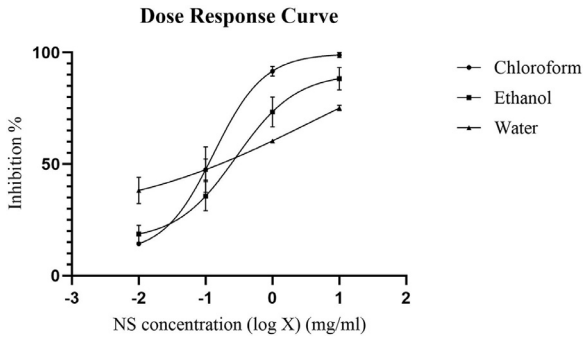

Figure 3. Dose-dependent S1-ACE2 inhibition with chloroform, ethanol, and water extracts.

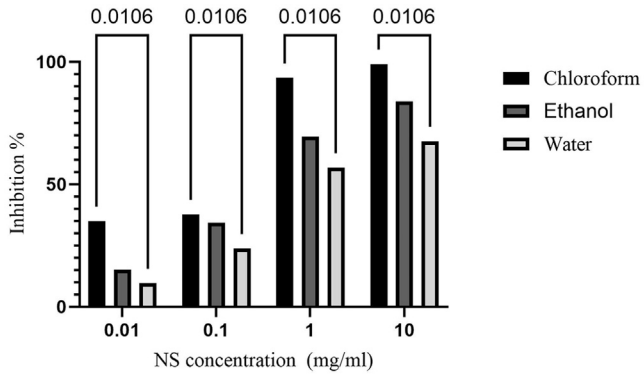

Figure 4. Inhibition % compared between chloroform, ethanol, and water extracts.

Discussion

The fundamental interaction between the S1 subunit of viral S-protein and the target cell surface receptor, ACE2, facilitates SARS-CoV-2 virus entrance into target cells.[15] Therefore, the ACE2 receptor is a key target for SARS-CoV-2 entrance inhibition.[8] Several clinical studies have demonstrated the efficacy of NS treatment in COVID-19 patients helping with faster symptom recov-

ery, viral clearance, and fewer sequelae.[11–13] While acute respiratory distress syndrome (ARDS) and cytokine storms caused severe mortality in COVID-19 patients, the anti-inflammatory and immunomodulatory effects of *N. sativa* suggest potential therapeutic benefits. Therapeutic properties of NS also include improvement in eosinophil counts and serum IgE levels, modulation of immune system activity and stimulation of humoral and cellular immunological responses. Furthermore, NS have been demonstrated to relax tracheal smooth muscle, thereby improving pulmonary function test (PFT) results in obstructive lung illnesses like asthma.[16]

Numerous molecular docking studies have demonstrated that NS components such as TQ, nigellidine, and beta-bisabolene can impede S-ACE2 interaction. Apart from ACE2, these docking experiments could point to several binding sites for NS components to SARS-CoV2 viral proteins and host proteins.[7,9] Although a recent *in vitro* study demonstrated the affinity of TQ, a major phytoconstituent in NS seeds, for ACE2[10] no study has been reported on the effect of NS extracts on the S1-ACE2 interaction. Other extracts such as rose and pomegranate have also proved its effectiveness against spike protein-ACE2 interaction.[17,18] In this study, we focused on the potential inhibitory effect of NS on the S1-ACE2 interaction through the SARS-CoV-2: S1 Protein-ACE2 Binding Inhibitor Screening Kit. We observed that the extracts inhibited the S1-ACE2 interaction in a dose-dependent manner. The IC50 values for chloroform, ethanol and water extracts were found to be 0.132 mg/ml, 0.288 mg/ml and 4.06 mg/ml, respectively. This shows the potential ability of NS extracts to block the attachment of the S1 subunit of the SARS CoV2 virion to the ACE2 host cell receptors, thereby providing protection against viral infection. This is critical because, while the COVID-19 pandemic has been contained internationally, the battle continues as other strains emerge. As a result, the mechanisms of NS against SARS-CoV-2 are worth investigating owing to their natural origin and due to their postulated effectiveness in prophylaxis and treatment of viral infections. The strength of this research lies in the presence of phytochemicals in the chemical fingerprint which further indicates that S1-ACE2 inhibition could be a result of the active components in the NS extracts. However, additional screening studies with extract fractions are required to determine which constituents exhibit this action and their inhibition mechanism. Chloroform extracts nonpolar compounds from herbal material. The higher effectiveness of chloroform extract in the current study could be due to the presence of nonpolar components such as thymoquinone. Furthermore, the limitation of this study comprises of the fact that preliminary *in vitro* studies are required to confirm that the ACE2 receptor inhibition does not alter the normal physiological functions of ACE2.

Conclusion

Our results showed that NS extracts inhibit the interaction between the S1 subunit of the SARS-CoV2 spike protein and ACE2 *in vitro. The* highest S1-ACE2 inhibition of 98.9% at 10 mg/ml concentration of NS chloroform extract may be due to the presence of nonpolar phytochemicals such as thymoquinone, thymohydroquinone, and nigellidine in the chloroform extract. In addition, S1-ACE2 inhibition showed a steady increase with increasing concentrations of 0.01, 0.1, 1, and 10 mg/mL for each of the three NS extracts (chloroform, ethanol, and water).

Ethical statement

Organic Nigella sativa seeds, selected from Imtenan Health Shop, Obour City, Egypt, were used in this research study.

Author contribution

Conceptualization: N.M.A., A.C.C., and R.R.; Methodology: N.M.A., A.C.C., and R.R.; Validation: R.R. and A.C.C.; Formal Analysis: R.R., A.C.C., and N.M.A.; Investigation: N.M.A., A.C.C., and A.P.; Data Curation: N.M.A. and A.C.C.; Writing—Original Draft Preparation: N.M.A. and A.C.C.; Writing—Review & Editing: R.R., A.C.C., N.M.A., N.K., C.G.V., R.K.J., A.P., H.A., F.A., S.I., F.N., Y.L.; Visualization: N.M.A. and A.C.C.; Supervision: R.R. and A.C.C.; Project Administration: A.C.C.; Funding Acquisition: R.R.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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