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Antiviral activity of *Nigella sativa* oil against two SARS-CoV-2 surrogates *in vitro* as a dietary product

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Submission Date: July 22nd, 2024; **Acceptance Date:** August 28th, 2024; **Publication Date:** September 16th, 2024

Please cite this article as: Huang J., Jiang X., Wang J. Antiviral activity of Nigella sativa oil against two SARS-CoV-2 surrogates in vitro as a dietary product. *Functional Foods in Health and Disease* 2024; 14(9):653-662.
DOI: https://doi.org/10.31989/ffhd.v14i9.1411

ABSTRACT

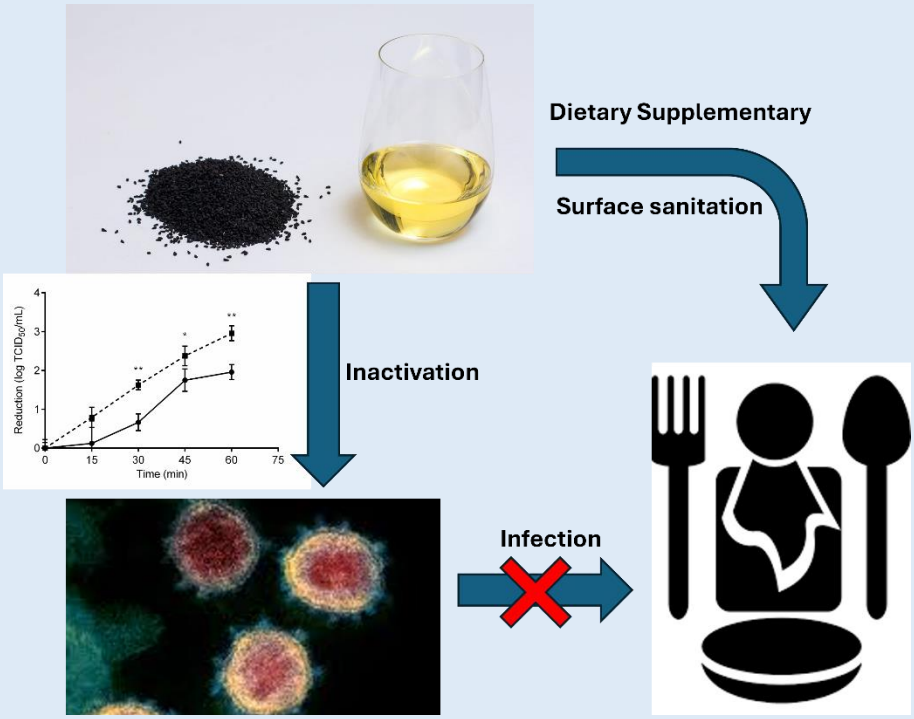
Background: Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is primarily transmitted via aerosol and droplets, but environmental surfaces or potentially contaminated foods may contribute to further viral transmission. *Nigella sativa* seed (black cumin seed) oil possesses various biological activities against bacteria, fungi, parasites, or viruses.

Objectives: This study aimed to explore black cumin seed oil (BSO) with anti-SARS-CoV-2 activities using bovine coronavirus (BCoV) and human coronavirus (HCoV) OC43 as surrogates.

Methods and Results: BSO was tested at 1-100 mL/L concentrations for cytotoxicity towards human rectal tumor (HRT-18G) cells and 0.1-10 mL/L for efficacy against BCoV and HCoV OC43 in suspension. Two coronaviruses were separately mixed with BSO suspension and incubated for various times (0, 15, 30, 45, and 60 min) at 4, 23, and 37°C. The virus titer reduction was determined by median tissue culture infectious dose ($TCID_{50}$) assay with HRT-18G cells as the host. BSO concentrations of 1-20 mL/L exhibited low cytotoxicity (<10%) towards HRT-18G cells. The inactivation of coronaviruses by BSO was concentration-, temperature-, and exposure time-dependent. At 37°C, BSO reduced 2.0 logs of BCoV and 3.0 logs of HCoV OC43 in 60 min, respectively. Specifically, half maximal effective concentration (EC_{50}) of BSO at 4, 23, and 37°C after the 1-h exposure was 4.28, 3.06, and 0.87 mL/L against BCoV, respectively, and 0.61, 0.61 and 0.48 mL/L against HCoV OC43, respectively. Moreover, BSO didn't affect the attachment of BCoV and HCoV OC43 into host cells.

Conclusion: Our findings on the inhibition activity of BSO against SARS-CoV-2 surrogates suggested the potential use of this natural product in mitigating SARS-CoV-2 infection. Our results also suggest that HCoV OC43 may be a more appropriate surrogate for SARS-CoV-2 to screen antiviral dietary products.

Keywords: *Nigella sativa,* SARS-CoV-2 surrogates, antiviral activity, quantitative suspension test, dietary supplement



INTRODUCTION

Since 2019, over 700 million cases of illness and nearly 7 million deaths worldwide have been attributed to the high transmissibility and severity of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which has spawned the development of various vaccines to curb its spread [1, 2]. However, the ongoing mutation of SARS-CoV-2 limited the sustained effectiveness of the available vaccines [3]. SARS-CoV-2 is primarily transmitted by aerosol and droplets, with viable SARS-CoV-2 found in environments. The persistence of SARS-CoV-2 in the environment suggests that SARS-CoV-2 could also be transmitted via environmental surfaces or potentially contaminated foods [4, 5]. In addition, SARS-CoV-2 was also found in stool samples, suggesting potential transmission from the contaminated water or surfaces to foods [6]. Similar to influenza, coronavirus is most likely here to stay as a seasonal virus. Therefore, effective prevention of SARS-CoV-2 infections should be prioritized.

A few medicines (e.g., remdesivir, nirmatrelvir) have been reported effective against SARS CoV-2 infections, but along with various toxicity to humans [7]. Thus, herbs with low toxicity traditionally used as a dietary supplement might be alternatives to therapeutic treatments to prevent or heal COVID-19 infections [8]. As 83 compounds from natural products have been found to possibly be effective against SARS-CoV-2, several compounds were found in *Nigella sativa* seed (black cumin seed) oil (BSO) with various biological functions

against bacteria, fungi, parasites, or viruses [8-10]. BSO reportedly inactivated the enveloped viruses *in vitro*, such as the Laryngotracheitis virus, suggesting its potential viricidal activity against SARS-CoV-2 [9]. Thymoquinone, as the main active ingredient of BSO, has antioxidant activity contributing to antiviral activity, which can directly damage viral capsids [11]. Moreover, both BSO and thymoquinone have been found to induce an immunological response to reduce viral loads and mitigate symptoms in animal models and clinical trials [12, 13]. Although BSO significantly reduced the pathogenesis of SARS-CoV-2 in a clinical trial [12], no study has yet determined the anti-SARS-CoV-2 activities of this natural product.

Considering the extreme contagiousness of SARS-CoV-2, *in vitro* studies on this virus are usually conducted using similar viruses with lower biosafety risks [14, 15]. While bacteriophage phi6 is a commonly used surrogate for the enveloped viruses, human coronaviruses (HCoV) NL63, 229E, and OC43, and bovine coronavirus (BCoV) are more closely related to SARS-CoV-2, as they all belong to the *Coronaviridae* family [15, 16]. The U.S. Environmental Protection Agency allows using HCoV 229E and other coronaviruses for the registration of disinfectants effective against SARS-CoV-2 [17], but HCoV 229E was found harder to cause cytopathogenic effect than HCoV OC43, suggesting the difficulty of using HCoV 229E as the surrogate of SARS-CoV-2 [18, 19]. Since HCoV 229E and NL63 belong to a different genus than SARS-CoV-2, surrogates from the same genus, such as HCoV OC43 and BCoV, were proved more appropriate for *in vitro* studies of this virus [18, 20].

In this study, we evaluated the cytotoxicity of BSO and its antiviral activities against two SARS-CoV-2 surrogates, HCoV OC43, and BCoV, in suspension to explore the potential use of BSO as a dietary supplement to prevent or reduce SARS-CoV-2 transmission.

MATERIALS AND METHODS

Virus propagation and assays: The cell line and virus were cultured as previously described [19]. Human rectal tumor (HRT-18G) cells (ATCC CRL-3609) were cultured in complete medium, which was Dulbecco's Modified Eagle Medium (DMEM) supplemented with glucose (4.5 g/L), low-endotoxin heat-inactivated fetal bovine serum (FBS; 3%), penicillin (100 U/L), and streptomycin (100 mg/L), at 33°C inside a CO_2 incubator. HRT-18G cells at 90% confluence were infected with bovine coronavirus (BCoV) strain Mebus (BEI, NR-445) or HCoV OC43 (ATCC, VR-1558) at a multiplicity of infection (MOI) of 0.01 and incubated at 37°C. After a 5-day incubation, three freeze-thaw cycles were conducted to lyse cells and harvest BCoV and HCoV OC43, followed by centrifugation for 10 min at 5,000 ×*g* and 4°C. Approximately 10^8 median tissue culture infectious doses ($TCID_{50}$)/mL of BCoV and HCoV OC43 stocks were aliquoted and stored at -80°C. HRT-18G cells were passaged less than 30 times.

$TCID_{50}$ assays were performed to quantify viable BCoV and HCoV OC43 as previously described with modifications [21]. In brief, 90% confluent monolayers of HRT-18G cells were infected with 100 µL of samples at 37°C for 1 h inside a 5% CO_2 incubator, followed by adding 100 µL of infection medium, which was the complete medium with reduced FBS level (3% to 2%). After incubating at 37°C in a 5% CO_2 incubator for seven days, the virus titer was determined and calculated by the improved Kärber method [22]. To test cell line permissiveness and contamination, the BCoV or HCoV OC43 stock and phosphate buffer saline (PBS) were used as positive and negative controls, respectively.

Determination of cytotoxicity of BSO to the host cell line: Cold-pressed BSO (Alive Herbals, NY, U.S.) with a thymoquinone content of 0.95% was procured from a store and tested in the study. In addition to thymoquinone, BSO also contained other major compounds such as *p*-cymene, α-terpinene, carvacrol, and thymol [23]. The cytotoxicity of BSO on HRT-18G cells was tested via CyQUANT™ LDH Assay kit Cytotoxicity (Invitrogen, CA, U.S.). Briefly, 5,000 HRT-18G cells/100 µL

medium were inoculated in 96-well plates and incubated at 37°C, 5% CO_2 incubator overnight to simulate body temperature. BSO was diluted in 0.5% Tween-80 solution to concentrations of 1, 5, 10, 20, 50, and 100 mL/L. Ten microliters of each dilution were directly inoculated in 96-well plates with HRT-18G cells and incubated at 37°C, 5% CO_2 incubator for 1 h. Meanwhile, 10 µL of lysate solution provided by the kit and sterile water were incubated with HRT-18 cells as maximum lactic acid dehydrogenase (LDH) release control and spontaneous LDH release control, respectively. PBS and 0.5% Tween-80 served as diluent control. Afterward, 50 µL of supernatants were transferred into a new 96-well plate, and LDH activity was measured by a microplate reader (µQuant, Bio-Tek, VT, U.S.) following the kit instruction.

Quantitative suspension test: Six concentrations (0, 0.1, 0.5, 1, 5, and 10 mL/L) of BSO were tested against BCoV and HCoV OC43 in suspension as previously described [24]. Both BCoV and HCoV OC43 were prepared at a titer of approx. 6.0 $\log_{10}$ $TCID_{50}$/mL in 5% FBS solution. To test BSO, 100 µL of prepared viruses were mixed with 100 µL of BSO and incubated at 4, 23, and 37 °C for 1 hour, respectively. After the incubation, 200 µL of infection medium was added to neutralize products. Virus control was prepared by mixing 100 µL of viruses with 100 µL of PBS + 0.5% Tween-80 and incubating under the same conditions. To evaluate the effect of contact time, 10 mL/L of BSO was inoculated with two surrogates at a titer of ca. 6.0 $\log_{10}$ $TCID_{50}$/mL at 37 °C for 0, 15, 30, 45, and 60 min, respectively, then neutralized as described above.

Neutralization effectiveness controls were prepared by adding the 10 µL of virus suspension at a titer of ca. 6.0 $\log_{10}$ $TCID_{50}$/mL into 990 µL of the mixture of the BSO and infection medium. Samples, including controls and inhibition testing groups, were 10-fold serially diluted and measured via $TCID_{50}$ assay.

Determination of infection interference on the propagation of coronaviruses by BSO: To determine the anti-coronavirus mechanism of BSO, BSO at a concentration of 10 mL/L was tested against BCoV and HCoV OC43. Fifty microliters of prepared viruses at approx. 2×10^5 $TCID_{50}$/mL with 5% FBS were mixed with 50 µL of BSO and incubated with a monolayer of HRT-18G cells at 90% confluency in a 12-well plate at 37 °C for 1 hour, and the MOI was 0.01. After the incubation, 1 mL of Hanks' balanced salt solution was added to each well to wash off unattached viruses twice. Afterward, 1 mL of the infection medium was added to maintain the cell line, and the 12-well plate was immediately frozen three times to harvest attached viruses. Samples were 10-fold serially diluted and determined via $TCID_{50}$ assay.

Statistical analysis: A total of six replicates were tested in two independent quantitative suspension tests to determine half maximal effective concentration (EC_{50}) values at different temperatures, while triplicates were used to determine the cytotoxicity of BSO and the effect of exposure time on the antiviral activity. Log_{10} $TCID_{50}$ reductions were calculated by $\log_{10}$ (N_0/N_d), where N_d is the average coronavirus titers from the treatment samples and N_0 is the average coronavirus titers from the control samples.

Statistical analysis was performed using a one-way multiple-comparison ANOVA to determine the relationship between antimicrobials and log reduction. A non-linear regression, 4PL sigmoidal model, was used to calculate EC_{50} values. All results were expressed as mean ± standard deviation. All data were statistically analyzed using GraphPad Prism 6.01 (GraphPad Software, Inc., CA, USA), and a *p*-value of <0.05 was used to define a statistical significance.

RESULTS

Cytotoxicity of BSO on HRT-18 G cells: To evaluate the safety of BSO, the cytotoxicity of BSO was evaluated on HRT-18G cells. Cell viability in PBS and 0.5% Tween-80 was maintained at above 98.5% and 96.2%, respectively. Following 1-hr exposure, cell viability was 99.8, 98.9,

96.8, 91.3, 86.2, and 86.8% in the presence of black seed oil at 1, 5, 10, 20, 50, and 100 mL/L, respectively (Figure 1).

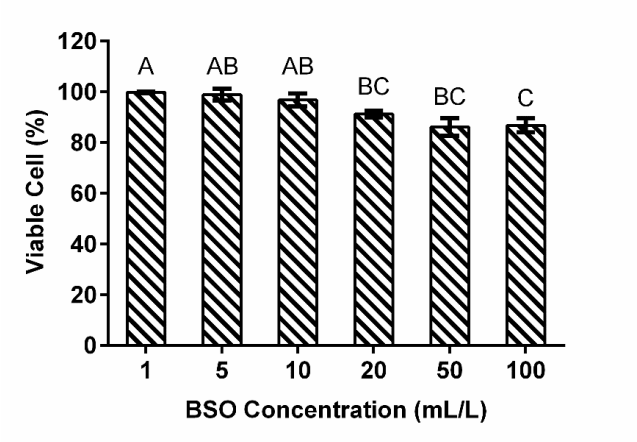


Figure 1. Cytotoxicity of BSO on HRT-18G cells. Error bars were from triplicates. Different letters (i.e., A, B, or C) represented a significant difference (p<0.05) in Tukey's test grouping.

The effect of exposure time on the antiviral activity of BSO against BCoV and HCoV OC43: To simulate the application of BSO as a dietary supplement, the effect of exposure time was studied at the body temperature, i.e. 37 °C. BSO inactivated more coronaviruses during the 1-h exposure, which reduced 0.1, 0.7, 1.8, and 2.0 logs of BCoV and 0.8, 1.6, 2.4, and 3.0 logs of HCoV OC43 following 15, 30, 45, and 60 min of exposure, respectively (Figure 2).

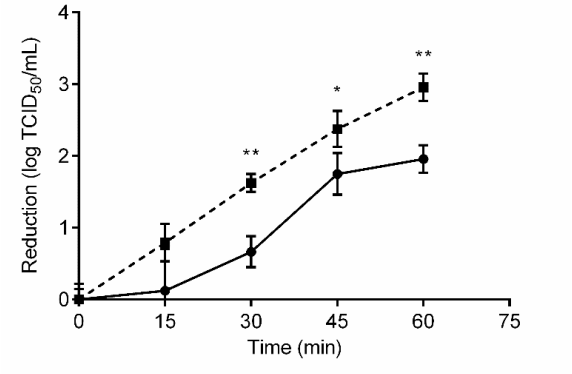


Figure 2. The effect of exposure time on the antiviral activity of BSO against BCoV (solid line + circles) and HCoV OC43 (dash line + squares). Error bars were from triplicates. The p-value between two coronaviruses for each time point was ≤0.05 (*) and ≤0.01 (**), respectively.

Antiviral activities of BSO against BCoV and HCoV OC43: To explore the potential use of BSO against SARS CoV-2 as a dietary supplement or surface disinfectant, the effect of BSO concentration at different incubation temperatures was evaluated. At 4°C, BSO at concentrations of 0.1, 0.5, 1, 5, 10 mL/L inactivated 1.42, 10.75, 23.50, 50.66, and 65.12% of BCoV and 17.95, 38.98, 55.81, 69.12, and 76.25% of HCoV OC43,

respectively (Figure 3). At 23°C, BSO increased activities against coronaviruses with 22.38, 27.59, 28.43, 70.67, and 82.2% reduction of BCoV and 24.99, 50.83, 88.02, 98.20, and 99.39% reduction of HCoV OC43, respectively. When temperature was increased to 37°C, BSO at concentrations of 0.1, 0.5, 1, 5, and 10 mL/L demonstrated a greater capacity for virus inactivation with 26.41, 33.29, 73.00, 99.02, and 99.72% reduction of BCoV, and 45.84, 74.43, 95.05, 98.02, and 99.83% reduction of HCoV OC43, respectively. Based on inactivation curves, EC_{50} of BSO against BCoV at 4, 23 and 37°C after the 1-h exposure was 4.28, 3.06, and 0.87 mL/L, respectively, while EC_{50} of BSO against HCoV OC43 was 0.61, 0.61 and 0.48 mL/L, respectively.

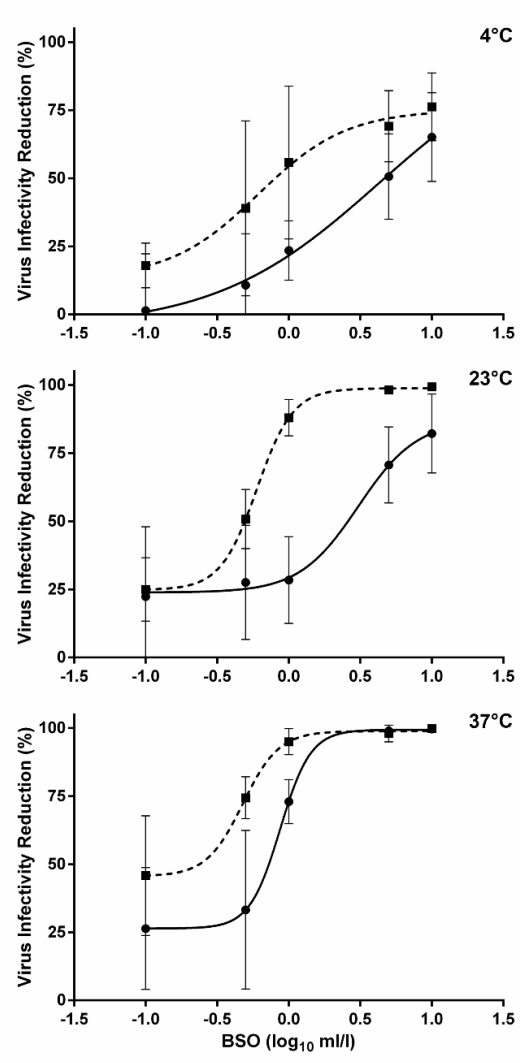


Figure 3. Effective concentrations of BSO that inhibit coronaviruses. The viruses were exposed to BSO for 1 h at 4°C (top), 23°C (middle) and 37 °C (bottom). Nonlinear regression was used to obtain EC_{50} values to BCoV (solid line + circles) and HCoV OC43 (dash line + squares). Error bars were from triplicates in each of the two independent experiments.

Infection interference on the propagation of coronaviruses by BSO: To explore the mechanism of BSO antiviral activity, infection interference on the propagation of coronaviruses by BSO was studied. After 1 hour of the infection, 2.1 $\log_{10}$ $TCID_{50}$ of BCoV and 2.8 $\log_{10}$ $TCID_{50}$ of HCoV OC43 attached to the host cell in controls. BSO at 10 ml/l didn't (p>0.05) affect the attachment as 2.4 $\log_{10}$ $TCID_{50}$ of BCoV and 2.7 $\log_{10}$

$TCID_{50}$ of HCoV OC43 were recovered (Table 1).

Table 1. Infection interference of BSO on the propagation of coronaviruses. Viruses were recovered 1 hour after the infection.

Treatment	Infectious virus population[a] (log_{10} $TCID_{50}$)	
	BCoV	HCoV OC43
Control	2.1±0.3[A]	2.8±0.2[A]
BSO (1%)	2.4±0.1[A]	2.7±0.1[A]

[a] The data are expressed as mean±SD. All data were collected in triplicates, and different letters (i.e., A, B) within the same column represented a significant difference (p<0.05) compared to the control.**Discussion**

Our studies revealed that BSO has low cytotoxicity on human HRT-18G cells and rapidly inactivated BCoV and HCoV OC43 *in vitro* within one hour, and the antiviral activity is BSO concentration-, temperature- and exposure time-dependent. Moreover, the antiviral activity of BSO was not due to the interference of coronavirus attachment to the host cells, probably as the result of directly damaging coronavirus structure.

Similar to a previous study with human epithelial cells, BSO was found with low cytotoxicity against human rectal tumor cells in our study [11]. In a clinical study, ingesting 500 mg of BSO twice daily for 10 days reportedly improved the percentage of patients recovered from mild COVID-19 [12], and the authors suggested the dual benefits, i.e. anti-inflammation and immunomodulatory effects of BSO on infection conditions [13]. Barakat et al. [27] studied the effect of BSO on the hepatitis C virus and reported the viral load of the hepatitis C virus was significantly decreased in patients who received 450 mg BSO three times/daily [28], indicating a potential viral load inhibition by BSO. In similarity, our study found BSO at a concentration of 10 mL/L, rapidly inactivated coronaviruses *in vitro*, suggesting a possible viral load inhibition effect on SARS-CoV-2 from BSO as dietary supplements besides inducing immunomodulation. Additionally, studies have shown that black cumin seed is safe to use as a functional food to reduce the risk of obesity, diabetes, and hypercholesterolemia [24-26]. The inactivation of coronaviruses by BSO was temperature-dependent *in vitro*, and the inactivation was much stronger at 37 °C than at 4 and 23 °C, suggesting the potential use as dietary supplements as reported for other natural products [24-27, 29]. However, due to fewer limitations for surface applications, black seed oil can also be formulated as a ready-to-use product for food or equipment surface protection.

The antiviral activities of BSO were attributed to its main active compounds, namely thymoquinone, *p*-cymene, α-terpinene, carvacrol, and thymol, which are also present in other plant extracts [30]. Thymoquinone, thymol, carvacrol, and *p*-cymene have been reported to exhibit antiviral activity against herpes simplex virus *in vitro* [30]. Although this study did not investigate the effect of a single bioactive compound, the high concentration of thymoquinone in BSO suggests that it may be the primary contributor to its antiviral properties.

The attachment of coronaviruses to host cells was generally driven by the interactions between coronavirus spike proteins and host cell acceptors [31]. The infection can be interfered with once the spike proteins are blocked by other chemicals [31]. An *in-silico* study predicted that phytoconstituents in BSO had greater affinity to SARS-CoV-2 spike proteins than the standard

drug hydroxychloroquine [32]. Based on our findings, BSO did not prevent coronaviruses from attaching host cells. The antiviral activity of BSO might be attributed to the ability of its active compounds in compromising the virus envelope or inhibiting the replication of coronaviruses in host cells, which needs further investigation.

HCoV OC43 was found more persistent in the cell culture medium with about 10% infectivity remaining after 4-day incubation at 37°C, compared to nearly 0% infectivity for HCoV 229E [33]. As HCoV 229E has some limitations as the SARS-CoV-2 surrogate, we chose both HCoV OC43 and BCoV as potential surrogates [19]. This is the first study to compare the sensitivity of HCoV OC43 and BCoV to dietary supplements. Our results have shown that BCoV was much less sensitive to BSO in suspension than HCoV OC43. As BCoV is specific for causing infections in bovine species, the use of HCoV OC43 may be a better surrogate for SARS-CoV-2 for evaluating the antiviral efficacy of dietary supplements.

CONCLUSION

Our findings have provided some valid data on the anti-coronaviruses by BSO, which at a concentration of 0.1-10 mL/L reduced both SARS-CoV-2 surrogates greater than 2 logs at body temperature. BCoV was less sensitive than HCoV OC43 to BSO. Although *in vitro*, our findings may indicate the potential use of BSO as a dietary supplement or food and equipment surface sanitizer to prevent COVID-19. Additionally, our results suggest that HCoV OC43 may be a more appropriate surrogate for SARS-CoV-2 to screen antiviral dietary supplement products.

Competing interests: The authors have no conflicts of interest to declare.

Author contributions: Conceptualization: X.J.; Methodology: J.H.; Formal analysis: J.H. and X.J.; Investigation: J.H.; Writing - original draft: J.H.; Writing - review & editing: X.J. and J.W.; Funding Acquisition: X.J; Supervision: X.J.

Acknowledgements and Funding: This research was financially supported by the South Carolina Department of Agriculture (SCDA) Agribusiness Center for Research and Entrepreneurship (ACRE) competitive grant program.

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