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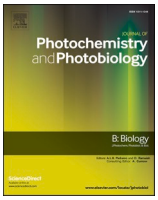

Pulsed blue light, saliva and curcumin significantly inactivate human coronavirus

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A B S T R A C T

In a recent study, we showed that pulsed blue light (PBL) inactivates as much as 52.3% of human beta coronavirus HCoV-OC43, a surrogate of SARS-CoV-2, and one of the major strains of viruses responsible for the annual epidemic of the common cold. Since curcumin and saliva are similarly antiviral and curcumin acts as blue light photosensitizer, we used Qubit fluorometry and WarmStart RT-LAMP assays to study the effect of combining 405 nm, 410 nm, 425 nm or 450 nm wavelengths of PBL with curcumin, saliva or a combination of curcumin and saliva against human beta coronavirus HCoV-OC43. The results showed that PBL, curcumin and saliva independently and collectively inactivate HCoV-OC43. Without saliva or curcumin supplementation 21.6 J/cm^2 PBL reduced HCoV-OC43 RNA concentration a maximum of 32.8% ($\log_{10} = 2.13$). Saliva supplementation alone inactivated the virus, reducing its RNA concentration by 61% ($\log_{10} = 2.23$); with irradiation the reduction was as much as 79.1%. Curcumin supplementation alone decreased viral RNA 71.1%, and a maximum of 87.8% with irradiation. The combination of saliva and curcumin reduced viral RNA to 83.1% and decreased the RNA up to 90.2% with irradiation. The reduced levels could not be detected with qPCR. These findings show that PBL in the range of 405 nm to 450 nm wavelength is antiviral against human coronavirus HCoV-OC43, a surrogate of the COVID-19 virus. Further, it shows that with curcumin as a photosensitizer, it is possible to photodynamically inactivate the virus beyond qPCR detectable level using PBL. Since HCoV-OC43 is of the same beta coronavirus family as SARS-CoV-2, has the same genomic size, and is often used as its surrogate, these findings heighten the prospect of similarly inactivating novel coronavirus SARS-CoV-2, the virus responsible for COVID-19 pandemic.

1. Introduction

With the recent outbreak of novel Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2), which, as of December 31, 2021, has infected more than 290 million, killed nearly five million and caused untold socio-economic devastation worldwide [1], research to advance the development and deployment of an antiviral agent capable of inactivating coronaviruses in the environment and in patient care scenarios has been on the rise. This has caused a resurgence of Photodynamic Inactivation (PDI), i.e., the use of light to excite a chemical photosensitizer to engender downstream inactivation of microorganisms. The first evidence that PDI effectively inactivates viruses emerged as early as the 1930s when Perdrau and Todd [2] used a combination of visible light and methylene blue to inactivate herpes virus, vaccinia virus, two avian viruses, canine distemper virus, and a host of other viruses.

With improvements in light technology and the emergence of relatively inexpensive light emitting diodes (LEDs), interest in PDI has been on the rise. Since the 1970s combinations of various spectra of light and photosensitizers have been used to inactivate a multitude of pathogens, including viruses that cause HIV-AIDS, Ebola, MERS-CoV, [3], hepatitis [4–6], Crimean-Congo hemorrhagic fever, and SARS. Other viruses susceptible to PDI include, Nipah virus [7], Zika virus [8], cytomegalovirus, human parvovirus B19, and human T-cell lymphotropic virus Types I and II [9–17]. Evidence from these studies show that PDI damages viral nucleic acid, resulting in fragmentation of the viral core [18,19], and that enveloped viruses, such as coronaviruses, are more susceptible to photo-damage than non-enveloped viruses [19–23]. The latter finding suggests that human coronaviruses, for example, HCoV-OC43, HCoV-229E and SARS-CoV-2, may be susceptible to PDI.

Studies have shown that even in the absence of a photosensitizer or an endogenous chromophore, certain wavelengths of light—besides ultraviolet light (UV)—can inactivate viruses. Violet-blue light inactivates baculoviruses [24], leukemia virus [25], feline calicivirus [26],

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HIV [27], herpes simplex [28] and the flu virus [29]. For example, murine leukemia virus exposed to blue 420–430 nm light lost significant infectivity compared to control viruses that were kept in darkness [25]. The antiviral effect was not due to UV which was eliminated by light filtration, neither was it due to the presence of an endogenous or exogenous chromophore which—if at all present—was experimentally neutralized. The loss of infectivity was shown to be light-induced impairment of the reverse transcription process [25]. Support for this finding comes from a recent work which showed that two surrogates of SARS-CoV-2, human beta coronavirus HCoV-OC43 and human alpha coronavirus HCoV-229E, are susceptible to 405 nm, 410 nm, 425 nm and 450 nm pulsed blue light (PBL) in the absence of a photosensitizer [30]. Irradiation with 425 nm PBL alone, using ultralow 12 mW/cm^2 irradiance and a dose of 130 J/cm^2 inactivated as much as 2.37 $\log_{10}$ (52.3%) HCoV-OC43 and 1.46 $\log_{10}$ (43.8%) HCoV-229E. Inactivation of both viruses was less at a lower irradiance and dose, suggesting that, perhaps at higher doses or with other ways of improving the protocol, 100% inactivation of the viruses may be attained [30].

Since certain photosensitizers potentiate the effect of light, it seems plausible that a combination of PBL and a blue light-absorbing photosensitizer will be more antiviral than light alone and may yield 100% inactivation of coronaviruses even at a relatively low irradiance and dose. Curcumin, the active ingredient in turmeric (*Curcuma longa L)* with a long history of being an antioxidant, anti-inflammatory, anticancer, and anti-diabetic [31], is a known blue light-absorber with peak absorption at 425 nm [32,33], and can act as a photosensitizer. Like violet-blue light, it is antiviral against several viruses, including hepatitis virus [34–38], influenza virus [39–42], herpes virus [43–45], human papilloma virus [46,47], cytomegalovirus [48,49], norovirus [50], as well as Zika virus [51,52], Dengue virus and chikungunya virus, [40,52–54], and human immunodeficiency virus (HIV) [55–59]. These findings suggest that curcumin could potentiate the antiviral effect of PBL.

In addition to curcumin, we tested the effect of saliva supplementation because of its known antiviral properties [60–63]. Its antiviral properties enable it to protect humanity from a wide range of infections [60]. Whole saliva from healthy donors neutralizes influenza A virus [61–63]. Further, it inactivates hantavirus [64], and its potential to inhibit SARS-CoV-2 has been suggested [65,66].

Since PBL, curcumin and saliva are independently antiviral, we studied the potential effect of combining various wavelengths of PBL with curcumin, saliva or a combination of curcumin and saliva against human beta coronavirus HCoV-OC43. PBL alone inactivates as much as 52.3% of the virus [30]; therefore, our specific aim was to determine if either or both supplements, curcumin and saliva, potentiates the antiviral effect of PBL compared to PBL alone.

First isolated more than 50 years ago, HCoV-OC43 is endemic in human populations, and is a major cause of annual epidemics of respiratory tract infections worldwide [67]. It is often used as a surrogate for SARS-CoV-2, the virus responsible for COVID-19 pandemic because, like SARS-CoV-2, it is a single stranded RNA virus transmitted by air and direct contact [68]. Moreover, it is of the same beta coronavirus genus as SARS-CoV-2 and has similar genomic size [69,70].

Considering the devastating effects of coronavirus pandemics, finding a way to inactivate viruses in humans and clearing the environment of viruses would be of immense benefit to mankind. Available treatments, such as vaccination, have enabled humanity to cope with coronaviruses without clearing the viruses from the environment. The end result has been that each wave of coronavirus disease eventually becomes endemic; then, over time subsequent outbreaks of related infections augment the burden of disease that humanity must contend with. For example, HCoV-OC43—a common cold coronavirus, which together with HCoV-229E are responsible for 15–30% respiratory tract infections every year, has been endemic in human populations for more than 50 years [67]. Similarly, SARS-CoV-1, the virus responsible for SARS which was first identified in February 2003 following a major outbreak in China [71], and MERS-CoV, the virus responsible the outbreak of MERS Disease in Saudi Arabia in 2012 [72], remain prevalent in various regions of the world. Clearing the environment of these viruses would be of immense benefit to mankind because it will reduce the burden of disease in terms of morbidity and mortality and lessen the devastating socio-economic consequences of disease epidemics and pandemics. Moreover, as a supplemental technology, PBL can help reduce viral load in humans and enable the patient's immune system to handle coronavirus infections.

2. Materials and Methods

2.1. Pulsed Blue Light Device

Custom-made PBL equipment, obtained from Carewear Corp., Reno, Nevada, USA, was used for this study. The light sources used were conventional high efficiency LEDs which were mounted on plastic bridges and were calibrated to produce an average irradiance of 12 mW/cm^2 with a peak irradiance of 36 mW/cm^2 at the surface of the virus samples. The average irradiance produced by each light source was factory calibrated using a Labsphere ILLUMIA PLUS 610–050-2Pi integrating sphere and a spectrometer to an accuracy of +/− 5%. Average irradiance values were measured at the surface of the viral samples with a calibrated Thorlabs PM100A – Compact power meter console. Each device emitted pulsed light either at 405 nm, 410 nm, 425 nm or 450 nm wavelength depending on the user's choice. At each wavelength, the spectral width was approximately 20 nm. The light was square wave pulsed at 33Khz with a 33% duty cycle—i.e., light emitted 33% of the time and off 67% of the time. The pulse duration was 10 microseconds with an off time of 20 microseconds. The irradiation parameters were selected based on prior studies [30].

2.2. Curcumin and Saliva Supplements

Water soluble Curcumin consisting of a mixture of N-Acetylcysteine – Curcumin complex and Tumeric Curcuminoids was obtained from Ultra Botanica LLC, Oklahoma City, Oklahoma, and was used for this study. Based on the recommendations and measured bioavailability in mouse models and human subjects provided by the manufacturer, we used a curcumin level of 10 micromolar in aqueous solution. We prepared the stock solution of curcumin by dissolving 14,720 g/L in sterile water. For each experiment, 70 μL of the stock solution was added to 140 μL of 5×10^3 PFU/mL virus. The curcumin supplementation experiment was intended to mimic the potential effect of PBL irradiation of the mouth following curcumin consumption.

The pharmaceutical grade artificial saliva (pH of 6.8) was obtained from Pickering Laboratories Inc., Mountain View, California. For each experiment, 70 μL of saliva was mixed with 5×10^3 PFU/mL of the virus and irradiated with PBL. When combined with curcumin, 70 μL of 10 μM solution of curcumin and 70 μL of saliva were added to 140 μL of 5×10^3 PFU/mL virus and then irradiated with PBL. The saliva supplementation experiment was intended to mimic the potential effect of PBL in the oral cavity.

2.3. HCoV-OC43 Irradiation

Viral titers of 5×10^3 PFU mL^{-1} of HCoV-OC43 coronavirus, obtained from the American Type Culture Collection (Beta coronavirus 1, ATCC® VR1558™), were used for the experiment. Aliquots of 140 μL of 5×10^3 PFU mL^{-1} of the virus were then transferred into different six well plates and supplemented with either 70 μL (10 μM) of curcumin, 70 μL of artificial saliva, or a combination of 70 μL curcumin and 70 μL artificial saliva. Plates were then irradiated with either 405 nm, 410 nm, 425 nm or 450 nm PBL, using 21.6 J/cm^2 fluence at 12 mW/cm^2 irradiance. The control group was neither irradiated nor supplemented with a photosensitizer (Table 1). Each set of experiment was repeated three

Table 1
Experimental Groups.

Group	Irradiation (Fluence = 21.6 J/cm^2; irradiance = 12 mW/cm^2)
HCoV-OC43 Without Supplementation *(Control group)*	None
HCoV-OC43 Without Supplementation	405 nm, 410 nm, 425 nm or 450 nm
HCoV-OC43 With Saliva Supplementation	None
HCoV-OC43 With Saliva Supplementation	405 nm, 410 nm, 425 nm or 450 nm
HCoV-OC43 With Curcumin Supplementation	None
HCoV-OC43 With Curcumin Supplementation	405 nm, 410 nm, 425 nm or 450 nm
HCoV-OC43 With Saliva and Curcumin Supplementation	None
HCoV-OC43 With Saliva and Curcumin Supplementation	405 nm, 410 nm, 425 nm or 450 nm

times; then viral RNA was extracted using a Qiagen RNA extraction kit, and quantitated using a Qubit fluorometer and RT-LAMP as detailed below.

2.4. Viral Nucleic Acid Extraction

RNA from irradiated and non-irradiated viruses were extracted using the QIAamp viral RNA kit (QIAGEN, Valencia, CA). Briefly, samples (approximately 140 μL) were placed in separate DNase/RNase free 1.5 mL Eppendorf tubes and lysed under highly denaturing conditions by adding 560 μL of lysis buffer to inactivate RNases and to ensure isolation of intact viral RNA. The mixture was pulsed-vortexed for 15 s, incubated at room temperature for 10 min, and briefly centrifuged to remove drops from the lid of the tubes. Then, 560 μL of 96–100% ethanol was added and pulsed vortexed for another 15 s. Then, 630 μL of the samples were loaded onto the QIAamp Mini spin column containing a silica-based membrane. The spin columns were centrifuged at 6000 x*g* (8000 rpm) for 1 min and the filtrate discarded. The rest of the sample was then added to the column, centrifuged at the same speed for 1 min and the filtrate discarded. This process allowed the RNA to bind unto the membrane; contaminants were then washed away by adding 500 μL wash buffer 1 and centrifuging at 6000 x g (8000 rpm) for 1 min. A second 500 μL wash buffer (wash buffer 2) was then added to the column and centrifuged at full speed (20,000 x g, 14,000 rpm) for 3 min. The column was then placed in a clean DNase/RNase free 1.5 mL micro-centrifuge tube and 40 μL of buffer AVE (RNase-free water containing 0.04% sodium azide, elution buffer) added and allowed to equilibrate at room temperature for 1 min before centrifuging at 6000 x g (8000 rpm) for 1 min. Another 40 μL of buffer AVE was added to the column and the elution process repeated. The high-quality RNA eluted, was quantitated immediately using the Qubit fluorometer or LAMP PCR; alternatively, it was stored at -20 °C for use later.

2.5. Fluorometric Determination of RNA Concentration

Two microliters of extracted RNA from both irradiated and non-irradiated groups were separately placed into clear thin-wall 0.5 mL PCR tubes containing 198 μL of Qubit working solutions. Qubit RNA standards in tris-EDTA (TE) buffer, were prepared separately by adding 10 μL of standard 1, to 190 μL of working solution and 10 μL of standard 2, to 190 μL working solution, after which each standard was inserted into the Qubit fluorometer for standard readings. Each sample tube was then inserted to determine their respective RNA concentration in ng/mL and percent RNA concentration computed with reference to the non-irradiated controls. Experiments were repeated four times with each treatment.

2.6. WarmStart RT-LAMP Assay

The WarmStart RT-LAMP test is an ultrasensitive assay based on a one-step Loop-Mediated Isothermal Amplification (LAMP) of RNA targets. It has been used to detect HCoV-OC43 [30] as well as SARS-CoV-2 [73,74]. The assay uses isothermal amplification techniques that provide rapid detection of a target nucleic acid, using LAMP-specific primers and a strand-displacing DNA polymerase. The kit (New England Biolabs) contains the WarmStart LAMP 2× master mix, which is a blend of BST 2.0 WarmStart DNA polymerase and WarmStart RTx (reverse transcriptase) in an optimized LAMP buffer solution. Eppendorf tubes were used for the assay. Briefly, each experimental tube, contained a set of six primers (below) for the gene of interest, 12.5 μL WarmStart LAMP Master Mix, 1.5 μL fluorescent dye, 2.5 μL (10×) LAMP Primer Mix, 2 μL of target RNA, and the remaining volume made up to 25 μL with H_2O. The tubes were then placed in the Rotor-Gene Q thermocycler (QIAGEN) and set at 65 °C for 30 min to perform the one-step reverse transcription to cDNA and real-time PCR of viral nucleocapsid gene using the following primers:

2.7. HCoV OC43

OC43_F3: AGT CCC AGC TAC TGA AGC T; OC43_B3: TGA CAT CAG CCT GGT TGC; OC43_ FIP: GGC AGC AGT TGA CGC TGG TT- GGG GTA CTG GTA CAG ACA CA; OC43_BIP: ACT ATC TGG GAA CAG GAC CGC A-C GAC CCA GTA GAC TCC GTT; OC43_F2: GGG GTA CTG GTA CAG ACA CA; OC43_B2: CGA CCC AGT AGA CTC CGT T.

The quantification cycle (Cq) value, i.e., the number of cycles required for the fluorescent signal to exceed the background fluorescence—also referred to as threshold cycle (Ct), crossing point (Cp), or take-off point (TOP)—was used to estimate the amount of RNA present in both irradiated and non-irradiated viruses; this corresponds to the amount of HCoV-OC43 present in the samples. Lower Cq values indicate high amounts of the target sequence. Higher Cq values signify lower amounts of target nucleic acid. In general, Cq values above 30 are deemed to represent non-detectable amounts of the target RNA.

2.8. Data Analysis

RNA concentrations were tabulated, summarized and subjected to inferential statistical analysis. Using wavelength, treatment group, irradiation dose as factors for each virus, a multivariate analysis of variance (and where appropriate, ANOVA) was used to compare outcomes and identify differences between groups, followed by Bonferroni *post-hoc* procedure to pinpoint differences between control and treated samples based on each factor. Data are graphed to ease interpretation and visualization. Statistical significance was set at $p < .05$; then ANOVA and Bonferroni post-hoc tests were used to ascertain differences between the RNA concentration of the irradiated and/or supplemented viral samples and the control non-treated samples.

3. Results

3.1. Effect of PBL Alone

At each of the four wavelengths, PBL irradiation without curcumin and/or saliva supplementation reduced HCoV-OC43 RNA concentration by 6.5% to 32.8% compared to 0% for the control group (Fig. 1, Tables 1 & 2). Irradiation with 425 nm and 450 nm wavelengths resulted in statistically significant reductions of viral RNA ($p < .05$); samples treated with 405 nm and 410 nm did not differ from control statistically ($p > .05$).

WarmStart RT-LAMP test affirmed the moderate effect of PBL irradiation alone, as evidenced by the amplification cycle of control and irradiated samples (Figs. 2 & 3, and associated tables). The two longer wavelengths, 425 nm and 450 nm, were most effective in inactivating

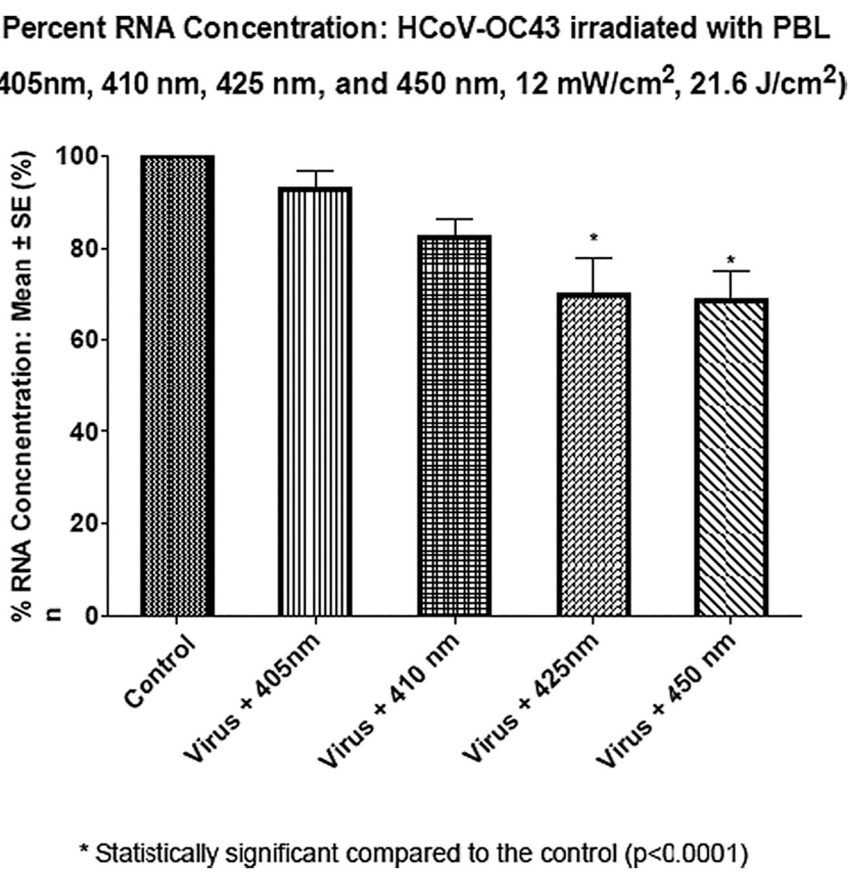


Fig. 1. The Effect of 405, 410, 425 and 450 nm PBL irradiation on HCoV-OC43 RNA. Each wavelength reduced RNA concentration; 425 nm and 450 nm wavelengths were more effective; * indicates statistically significant difference compared to control.

the virus, resulting in 31.9% ($\log_{10} = 2.13$) and 32.8% ($\log_{10} = 2.13$) reduction in viral RNA, respectively. Our previous report showed that at higher fluences, 32.5 J/cm^2 and 130 J/cm^2, 405 nm PBL inactivated 44.7% and 52.3% HCoV-OC43, respectively. Therefore, this finding that at a lower fluence, 21.6 J/cm^2, 405 nm PBL inactivated 6.5% of the virus supports the view that PBL is more antiviral at higher doses than lower doses [30].

3.2. Effect of Saliva Supplementation With or Without PBL

As shown in Fig. 4 and Table 2, saliva supplementation alone, significantly reduced percent RNA concentration to 39% ($\log_{10} = 2.23$; $p < .001$). This finding supports previous reports which show that saliva is antiviral [58–64].

Further, at each wavelength tested, the combination of saliva and PBL was more antiviral than PBL alone. Compared to 405 nm PBL irradiation alone, RNA concentration declined from 93.5% to 44.6% with saliva supplementation, and from 82.5% to 43.7% in saliva supplemented samples irradiated with 410 nm PBL. The corresponding data for 425 nm and 450 nm PBL were decreases of RNA concentration from 68.1% to 20.9% and from 67.2% to 21.9%, respectively; $p < .001$, Fig. 4; also see $\log_{10}$ values in Table 2). Irradiation of saliva supplemented samples with 425 nm and 450 nm wavelengths gave the best result. As detailed in Figs. 2 & 3, WarmStart RT-LAMP confirmed the antiviral effect of saliva with or without irradiation; however, the assay did not show a difference between the antiviral effect of saliva alone compared to saliva and irradiation at any wavelength tested, neither did it show a difference between the four wavelengths in saliva treated specimens ($p > .05$). This finding suggests that the fluorometric assay was more sensitive in differentiating treatment effect.

3.3. Effect of Curcumin Supplementation With or Without PBL

Curcumin alone reduced the concentration of viral RNA to 29.9% of control ($\log_{10} = 2.29$), yielding a better result than saliva alone. With irradiation, RNA concentration further declined to the range of 12.2% ($\log_{10} = 2.54$) to 15.3% ($\log_{10} = 2.34$) of control depending on wavelength. Irradiation with 405 nm, 410 nm, 425 nm and 450 nm wavelengths reduced RNA concentration to 15.3%, 13.1%, 12.2%, 12.7% of control, respectively ($p < .001$ in each case); 425 nm and 450 nm wavelengths inactivated HCoV-OC43 the most in curcumin treated samples (Table 2; Fig. 5). This finding suggests that as a photosensitizer, curcumin absorbs mostly light in the 425 and 450 nm spectra.

WarmStart RT-LAMP confirmed the superior antiviral effect of curcumin with or without irradiation compared to PBL treatment alone or treatment with PBL and saliva supplementation (Figs. 2 & 3). Indeed, the amplification cycle (Cq) and Take-Off values of curcumin supplemented samples with or without irradiation—each—exceeded 30, indicating that there were undetectable amounts of HCoV-OC43 RNA following curcumin supplementation with or without irradiation (Figs. 2 & 3). This finding corroborates our fluorometric data which showed over 87% reduction of viral RNA when curcumin supplemented samples were irradiated with 425 nm or 450 nm PBL. Since PBL and curcumin attack and fragment viral RNA, i.e., the core of the virus, it is possible that Qubit fluorometry, the assay used to detect the presence or absence of RNA in the samples, detected the mere presence of fragmented RNA. WarmStart RT-LAMP clearly showed that viral RNA was beyond detectable levels in PBL irradiated curcumin supplemented specimens.

In a separate study, we irradiated the curcumin supplemented virus with 12 mW/cm^2 irradiance and a much higher fluence of 130 J/cm^2 of 425 nm PBL, i.e., the wavelength which was most effective in inactivating the virus with curcumin supplementation. Our goal was to

Table 2
Summary Data Showing The Effect of Light, Saliva and/or Curcumin On HCoV-OC43.

Treatment	% RNA ± SE	% Inactivation ± SE	Log_{10}Reduction of RNA Concentration
HCoV-OC43 (Control)	100 ± 0.00	0	
HCoV-OC43 + 405	93.5 ± 4.43	7.1 ± 3.93	1.23
HCoV-OC43 + 410	82.5 ± 4.05	17.5 ± 4.05	1.67
HCoV-OC43 + 425	68.1 ± 7.39	31.9 ± 4.26	2.13
HCoV-OC43 + 450	67.2 ± 5.33	32.8 ± 3.07	2.13
HCoV-OC43 + Saliva	39.0 ± 8.02	61.0 ± 6.23	2.23
HCoV-OC43 + Saliva + 405	44.6 ± 5.76	53.4 ± 5.76	2.16
HCoV-OC43 + Saliva + 410	43.7 ± 2.72	56.3 ± 2.72	2.17
HCoV-OC43 + Saliva + 425	20.9 ± 2.48	79.1 ± 1.43	2.45
HCoV-OC43 + Saliva + 450	21.9 ± 2.92	78.1 ± 1.68	2.44
HCoV-OC43 + Curcumin	29.9 ± 8.91	70.1 ± 8.91	2.29
HCoV-OC43 + Curcumin +405	15.3 ± 2.20	84.8 ± 2.20	2.34
HCoV-OC43 + Curcumin +410	13.1 ± 1.13	86.9 ± 1.13	2.36
HCoV-OC43 + Curcumin +425	12.2 ± 5.19	87.8 ± 3.00	2.54
HCoV-OC43 + Curcumin +450	12.7 ± 5.38	87.3 ± 3.10	2.53
HCoV-OC43 + 405 + Curcumin + Saliva	16.6 ± 3.20	83.4 ± 3.20	2.34
HCoV-OC43 + 410 + Curcumin + Saliva	12.0 ± 2.15	88.0 ± 2.15	2.36
HCoV-OC43 + 425 + Curcumin + Saliva	9.8 ± 3.31	90.2 ± 1.91	2.56
HCoV-OC43 + 450 + Curcumin + Saliva	9.8 ± 3.61	90.2 ± 2.08	2.56

determine if the two assays would show non-detectable levels of viral RNA following irradiation at a higher fluence which would normally result in clearance of the virus. Curcumin concentration and irradiance were kept constant at 10 μM and 12 mW/cm^2, respectively. Treatment was carried out for 3 h in order to attain 130 J/cm^2 fluence. As expected, WarmStart RT-LAMP assay showed that viral RNA was beyond detectable level after treatment. The cq and take-off values were quite high, 57.63 and 61 respectively. Then, we used Qubit Flourometry to quantitate viral RNA in the samples. As shown in Table 3, the flourometric assay still showed 12% viral RNA concentration in the treated group, confirming our suspicion that the assay is so sensitive that it would show a percentage of remnant RNA even when RT-LAMP, the gold standard for viral RNA detection, revealed that viral RNA was beyond detectable level. Clinically, the RT-LAMP result would be interpreted as being "negative", i.e., no viral RNA detected.

3.4. Effect of Combined Saliva and Curcumin Supplementation With or Without PBL

PBL irradiation of samples supplemented with saliva and curcumin further enhanced the antiviral effect of PBL compared to irradiation of samples supplemented with curcumin alone. The combined effect of both supplements was not cumulative; it seemed synergistic, suggesting a common mechanism of action of the two supplements. Whereas saliva and curcumin each reduced viral RNA from 100% to 39% and 29.9% respectively, combining the two supplements further reduced RNA concentration to 16.6%; i.e., 13.3% better than curcumin supplementation alone. In other words, in terms of virus inactivation, saliva alone inactivated 61% of the virus, curcumin alone inactivated 70.1% of the virus; the combination of both supplements inactivated 83.4% of the virus, not 61% plus 70.1% which would have resulted in over 100% inactivation of the virus. At each wavelength, PBL irradiation of curcumin and saliva supplemented samples potentiated the combined antiviral effect of both supplements, reducing RNA concentration to the range 9.8% to 16.7% of control. Again, the most effective wavelengths were 425 nm and 450 nm (Figs. 6 & 7 and Table 2), indicating that these wavelengths more effectively potentiate the photosensitizing effects of curcumin. (See Figs. 8 and 9.)

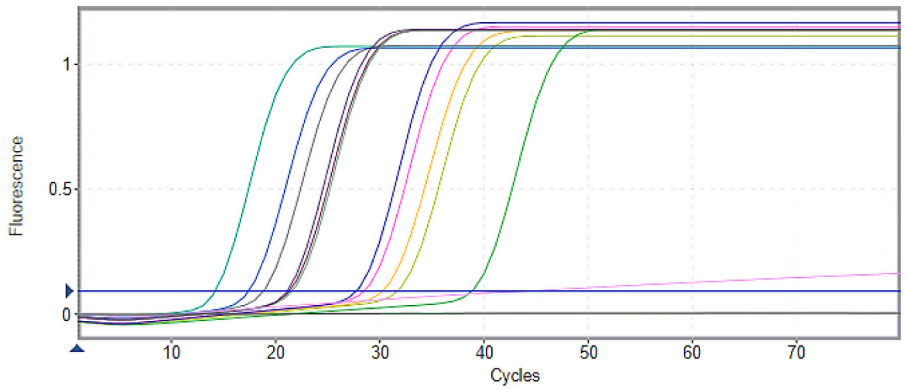
As shown in Figs. 2 and 3, WarmStart RT-LAMP affirmed the superior antiviral effect of PBL in viral samples supplemented with saliva and curcumin when compared to PBL alone or irradiation of saliva or curcumin supplemented samples (Figs. 2 & 3). Indeed, the amplification cycle of saliva and curcumin supplemented samples —with or without irradiation, exceeded 30. This finding indicates that there were clearly undetectable amounts of HCoV-OC43 RNA following saliva and curcumin supplementation with or without PBL irradiation (Figs. 2 & 3), and it corroborates our fluorometric data which showed up to 91.2% (Log_{10} = 2.56) inactivation of the virus when saliva and curcumin supplemented samples were irradiated with 425 nm or 450 nm PBL. Since the three treatments, PBL, saliva and curcumin—each—attack and fragment viral RNA, it is possible that the mere presence of fragmented RNA caused the fluorometric assay to show trace amounts of viral RNA. This view is supported by Warm Start RT-LAMP results which clearly showed that viral RNA was beyond detectable level.

4. Discussion

Our findings confirm that PBL alone is antiviral against human coronavirus HCoV-OC43, and that saliva and curcumin independently and mutually inactivate HCoV-OC43; moreover, the two supplements significantly complement the antiviral effect of PBL individually and collectively. Compared to control samples in which the change in RNA concentration was zero, without saliva or curcumin supplementation, PBL reduced HCoV-OC43 RNA concentration a maximum of 32.8% (log_{10} = 2.13). Saliva supplementation alone inactivated the virus, reducing its RNA concentration by 61% (log_{10} = 2.23); with irradiation the reduction was as much as 79.1%. Curcumin supplementation alone decreased viral RNA 71.1%, and a maximum of 87.8% with irradiation. The combination of saliva and curcumin reduced viral RNA 83.1% and decreased the RNA up to 90.2% with irradiation. Since the RNA is the main core of the virus, these results showing significant reductions in viral RNA clearly evince inactivation of the virus, more so because the two assays, Qubit fluorometry and qPCR – RT-LAMP, respectively quantitate and estimate viral nucleic acid.

The mechanisms by which PBL, curcumin and saliva inactivate viruses remain the focus of ongoing research works. It has been shown that violet/blue light inactivates viruses by disrupting the viral capsid and fragmenting viral nucleic acid, i.e., the viral core [25], particularly when used with a photosensitizer such as curcumin [75]. The antimicrobial effect of saliva seems related to its biochemical composition. Purified salivary proteins, such as histatins, human neutrophil defensins, MUC5B, and the scavenger receptor cysteine-rich glycoprotein 340 (GP340) have been shown to inhibit influenza virus A; GP340 also inhibits HIV [76]. Other components of saliva known to be antiviral include cathelcidin (LL-37), lactoferrin, lysozyme, mucins, peroxidase, salivary agglutinin (gp340, DMBT1), sIgA, SLPI, and α and β defensins [76]. These findings suggest that any or a combination of these biochemical components of saliva could be responsible for the observed antiviral effect of saliva against HCoV-OC43.

Curcumin has been shown to be antiviral against several viruses, including hepatitis virus [34,36–38,77], influenza virus [39–42], herpes virus [43–45], human papilloma virus [46,47], cytomegalovirus [48,49], norovirus [50], as well as Zika virus [51,52], Dengue virus and



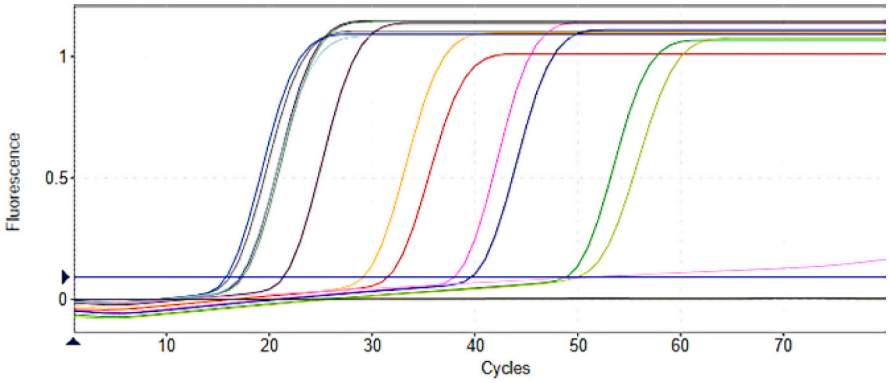
Sample Name	Cq	Color	Take-off
Non-irradiated Control (HCoV-OC43)	14.21		13.10
HCoV-OC43 + 405 nm	18.83		18.10
HCoV-OC43 + 410 nm	17.43		16.60
HCoV-OC43 + Saliva	21.33		21.30
HCoV-OC43 + Saliva + 405 nm	21.59		21.20
HCoV-OC43 + Saliva + 410 nm	21.12		20.50
HC0V-OC43 + Saliva + Curcumin	30.27		30.30
HC0V-OC43 + Saliva + Curcumin + 405 nm	28.51		28.40
HC0V-OC43 + Saliva + Curcumin + 410 nm	31.70		31.60
HCoV-OC43 + Curcumin	*		*
HCoV-OC43 + Curcumin + 405 nm	38.84		38.70
HCoV-OC43 + Curcumin + 410 nm	31.70		31.60

Fig. 2. RT-LAMP graph showing the effect of 405 and 410 nm PBL irradiation on HCoV-OC43 with or without supplementation with saliva or curcumin. (The Cq values, color legend and take-off are shown in the table accompanying the figure. Irradiation was carried out using PBL at 12 mW/cm^2, 21.6 J/cm^2; * denotes outlying data).

chikungunya virus, [40,52–54], and human immunodeficiency virus (HIV) [55–59]. The mechanisms involved appear to vary from one virus to another. For example, it impairs HIV infectivity by inhibiting integrase, the enzyme responsible for integrating HIV genome into the host DNA [56,57]. It inhibits gene expression and replication by down-regulating PGC-1a, a protein that co-activates gene transcription in hepatitis virus [36], and in influenza A virus it inhibits virus uptake, replication, and particle production [39,41,42,78]. These reports suggest that, like PBL and saliva, curcumin targets and fragments viral nucleic acids.

The results obtained from the two assays, Qubit Fluorometry and WarmStart RT-LAMP complement each other and support the view that the two supplements fragment viral nucleic acid. RT-LAMP detects the presence or absence of the virus, Qubit Fluorometry quantifies the presence or absence of viral RNA. The fluorometric assay revealed remnant (9.8% to 16.9%) RNA, WarmStart RT-LAMP showed that viral RNA was beyond detectable levels in virtually every curcumin or curcumin and saliva supplemented sample irradiated with PBL. Given the high sensitivity of both assays, it is possible that the remnant percentage of viral RNA detected by fluorometry represents fragmented RNA entities. Since Qubit fluorometry detects the presence or absence of RNA in the samples, it seems plausible that it would not have detected 100% clearance of RNA due to the mere presence of fragmented RNA. The WarmStart RT-LAMP results clearly showed that irradiation with PBL using curcumin or combined curcumin and saliva as supplements inactivates HCoV-OC43 to levels that are beyond detection, indicating that either there was no intact viral RNA whatsoever or that whatever was left was so minimal that it could not be detected by WarmStart RT-LAMP—the gold standard assay used to determine the presence or absence of coronavirus infection clinically.

Photodynamic inactivation using combined saliva and curcumin was slightly more effective than irradiation of samples supplemented with curcumin alone; however, the difference was not statistically significant ($p > .05$). The combined effect of both supplements was not cumulative but synergistic. This finding suggests a common mechanism of action of PBL and both supplements. Moreover, it supports results which show that all three—PBL, curcumin and saliva—inactivate viruses by fragmenting the viral core [24,25,39,41,42,61–63,66,75,78]. Whereas saliva and curcumin each reduced viral RNA from 100% to 39% and 29.9% respectively, combining the two supplements further reduced RNA concentration to 16.6%; i.e., 13.3% better than curcumin supplementation alone. In other words, in terms of virus inactivation, saliva



Group	Cq	Color Style	Take-off
Non-irradiated Control (HCoV-OC43)	17.07		16.30
HCoV-OC43 + 425 nm	16.18		15.40
HCoV-OC43 + 450 nm	15.83		14.90
HCoV-OC43 + Saliva	21.41		20.80
HCoV-OC43 + Saliva + 425 nm	17.47		16.80
HCoV-OC43 + Saliva + 450 nm	17.25		16.60
HCoV-OC43 + Saliva + Curcumin	29.16		28.90
HCoV-OC43 + Saliva + Curcumin + 425 nm	38.02		37.80
HCoV-OC43 + Saliva + Curcumin + 450 nm	39.70		39.60
HCoV-OC43 + Curcumin	31.56		31.00
HCoV-OC43 + Curcumin + 425 nm	48.88		49.00
HCoV-OC43 + Curcumin + 450 nm	50.02		51.20

Fig. 3. RT-LAMP graph showing the effect of 425 and 450 nm PBL irradiation on HCoV-OC43 with or without supplementation with saliva or curcumin. (The Cq values, color legend and take-off are shown in the table accompanying the figure. Irradiation was carried out using PBL at 12 mW/cm^2, 21.6 J/cm^2.

alone inactivated 61% of the virus, curcumin alone inactivated 70.1% of the virus; the combination of both supplements inactivated 83.4% of the virus, not 61% plus 70.1% which would have resulted in over 100% inactivation of the virus.

With or without supplementation, the two longer wavelengths, 425 nm and 450 nm, were most effective in inactivating the virus. Our previous report showed that at higher fluences, 32.5 J/cm^2 and 130 J/cm^2, 405 nm PBL inactivated HCoV-OC43 more—44.7% and 52.3%, respectively—and was more effective than 425 nm and 450 nm wavelengths [30]. Therefore, our finding that at a lower fluence, 21.7 J/cm^2, 405 nm PBL was less effective in clearing the virus than 425 nm or 450 nm may be due to the significantly lower irradiation dose used in the present study. Moreover, more virus was inactivated at the higher doses used in the previous study, and this supports the view that PBL is more antiviral at higher doses than lower doses [30].

With saliva and/or curcumin supplementation, the two longer wavelengths, 425 nm and 450 nm, also were consistently more effective in inactivating the virus than the two shorter wavelengths, 405 nm or 410 nm. This finding is consistent with the fact that as a photosensitizer, curcumin absorbs blue light mostly in the longer wavelength ranges used in this study. These same wavelengths potentiate the antiviral effect of curcumin more so than the shorter wavelengths, and this relates to the absorption spectra of curcumin with peak absorption in the range of 420 nm to 440 nm [32,33,79; Fig. 9]. The combination of saliva and curcumin supplements inactivated the virus more, with or without irradiation, and we ascribe this finding to the three factors—the antiviral effect of saliva, the photosensitizing effect of 425 nm and 450 nm light by curcumin, and the direct antimicrobial effect of PBL on the virus.

Our findings have promising implications in the ongoing effort to curtail coronavirus epidemics and pandemics. For example, with further development of this technology, two potential areas of application are patient care and environmental disinfection. Coronaviruses, such as HCoV-OC43 and SARS-CoV-2, typically invade the human body through the oro-nasal passages. Studies have shown that these viruses remain predominantly in these biomes and the upper airways during the initial few days of infection [80]. Consequently, each infected person can harbor and emit thousands of contagious viral particles into the environment in the form of large droplets and medium or small aerosol particles which can infect others or remain in the environment for a very long time. This makes the oral and nasal cavities ideal locations for safe inactivation of coronaviruses with PBL.

Irradiation of these cavities could clear or significantly reduce the viral load thereby enabling the patient's immune system to overcome the infection. Our work clearly shows that inactivation of the virus is possible in the oral cavity where PBL could reduce the viral load in the presence of natural saliva or curcumin and natural saliva. Curcumin is a ubiquitous food supplement—often referred to as the "golden spice." It has been a staple of human nutrition for thousands of years in several

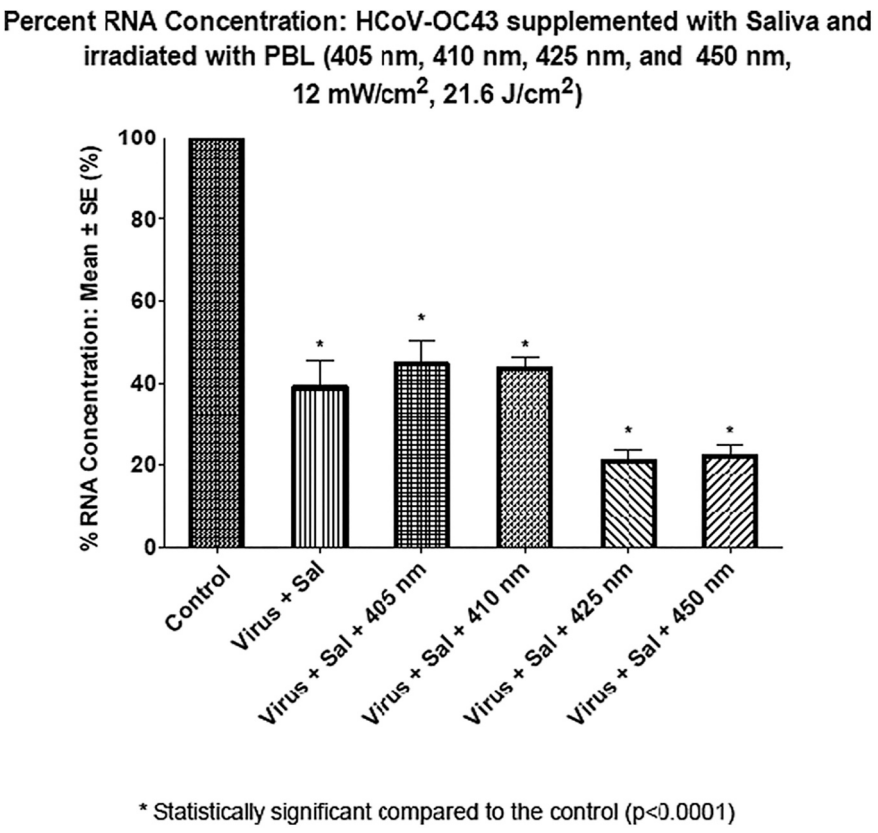


Fig. 4. The Effect of 405, 410, 425 and 450 nm PBL irradiation on saliva supplemented HCoV-OC43 viral samples. * Indicates statistically significant difference compared to control; there was a statistically significant reduction in RNA concentration at each wavelength tested, more so than PBL irradiation alone (sal = saliva).

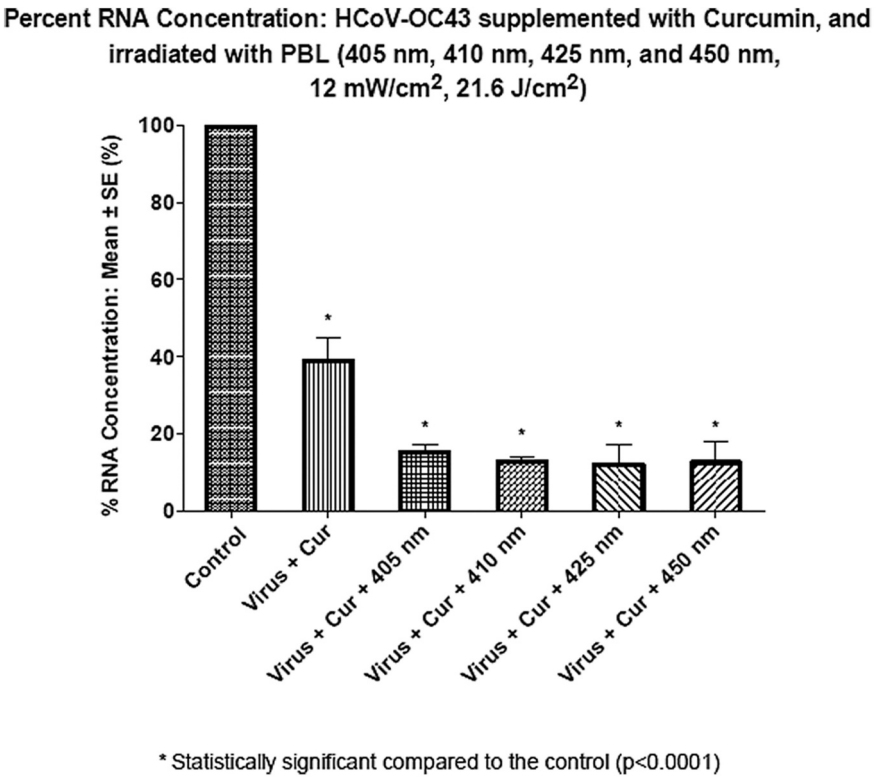


Fig. 5. The Effect of 405, 410, 425 and 450 nm PBL irradiation on curcumin supplemented HCoV-OC43 viral samples. * Indicates statistically significant difference compared to control; there was a statistically significant reduction in RNA concentration at each wavelength tested ($p < .001$), more so than PBL irradiation alone or irradiation of saliva supplemented samples (cur = curcumin).

Table 3
The antiviral effect of a higher fluence of 425 nm PBL (130 J/cm^2) on HCoV-OC43. Curcumin concentration and irradiance were kept constant at 10 μM and 12 W/cm^2, respectively. Treatment was carried out for 3 h in order to attain 130 J/cm^2 fluence. Whereas RT-LAMP showed that viral RNA was beyond detectable level, Qubit Flourometry still showed 12% RNA concentration.

Sample	RNA concentration (x10^3 ng/mL)	% inactivation	% RNA	Cq	Take-off
Non-irradiated Control (HCoV-OC43)	252	0	100	16.36	15.50
HCoV-OC43 + Curcumin +425 nm	30.8	88	12	57.63	61

regions of the world [31]. Therefore, as treatment for coronavirus infection, it should be easy to irradiate the oral cavity with an admixture of curcumin and natural saliva in consumers of curcumin. Furthermore, studies have shown that nasal secretion is antimicrobial too [81,82]. Even though we did not test the antiviral effect of PBL and nasal secretion, it seems reasonable to expect that irradiation of the nasal passages with PBL in the presence of mucous would result in significant inactivation of coronaviruses in the nasal cavity.

Blue light is innocuous and safe when used at appropriate doses. It has been used by dentists for surgery and to cure resins for decades without adverse effects. Further, PBL has been shown to eradicate bacteria [83–92]; therefore, in addition to reducing the viral load, irradiation of the oral and nasal passages with PBL could also clear the upper airways of the opportunistic bacteria that worsen coronavirus infections, and hence minimize the complications of infection.

For environmental disinfection, PDI with blue light presents several advantages. First, unlike sterilization with UV and chemical disinfectants, blue light is eco-friendly, and is better suited for disinfecting hard to reach spaces and crevices. Such spaces could be in homes, offices, schools, factories, and transportation systems, such as trains, cars, airplanes, and ambulances. Second, like SARS-CoV-2, HCoV-OC43 is highly contagious. Human to human transmission occur through viral droplets and particles which can survive on various surfaces and be airborne for hours. The particles are quite resistant to environmental conditions; this makes it readily transmissible. While available methods of limiting infection, such as social distancing, wearing of masks, efficient ventilation, air filtration of viral particles, and hand washing are effective in limiting person to person infection, they do not "kill" or inactivate viral particles, pulsed blue light does. Therefore, adding photo-disinfection to the clinical armamentaria currently used to limit coronavirus infections could be crucial in reducing the spread of disease.

5. Conclusions

Our findings affirm that pulsed blue light in the range of 405 nm to 450 nm wavelength is antiviral against human coronavirus HCoV-OC43, a surrogate of the COVID-19 virus, and one of the major strains of viruses responsible for the annual epidemic of the common cold. Further, it shows that with curcumin and/or saliva as a supplement(s) it is possible to photodynamically inactivate the virus to levels that are beyond detection using pulsed blue light. Since HCoV-OC43 is of the same beta coronavirus family as SARS-CoV-2, has the same genomic size, and is often used as its surrogate, these findings heighten the prospect of inactivating novel coronavirus SARS-CoV-2, the virus responsible for COVID-19, photodynamically with PBL.

Credit author statement

Chukuka Enwemeka: Conceptualization, Writing-Reviewing and

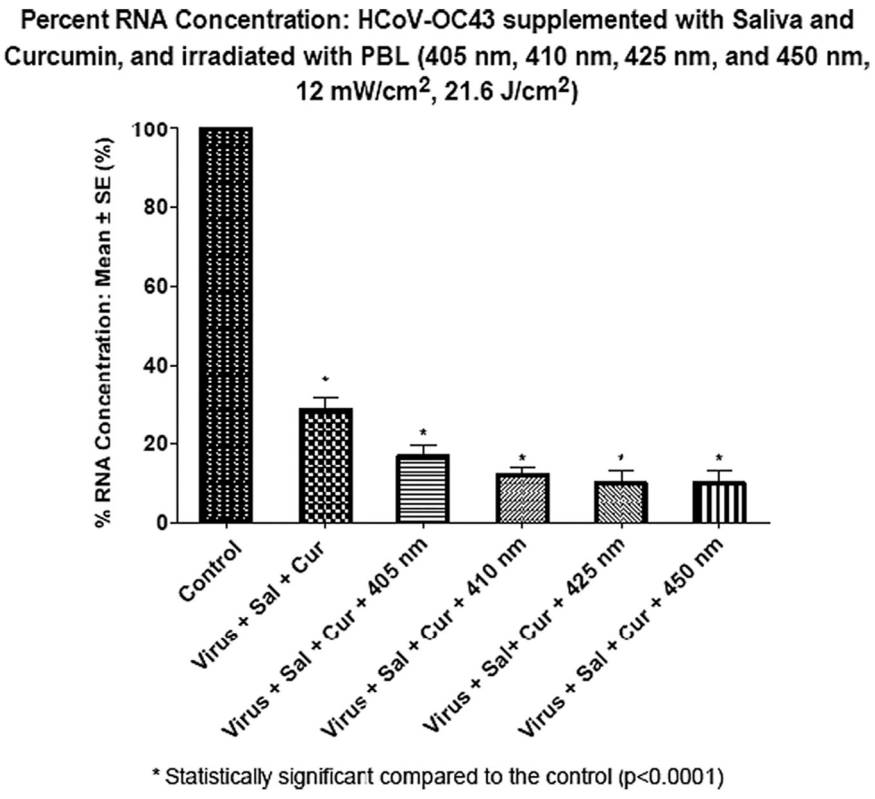


Fig. 6. The Effect of 405, 410, 425 and 450 nm PBL irradiation on saliva and curcumin supplemented HCoV-OC43 viral samples. * Indicates statistically significant difference compared to control; there was a statistically significant reduction in RNA concentration at each wavelength tested, more so than PBL irradiation alone, irradiation of saliva supplemented samples, irradiation of curcumin supplemented samples. (sal = saliva; cur = curcumin).

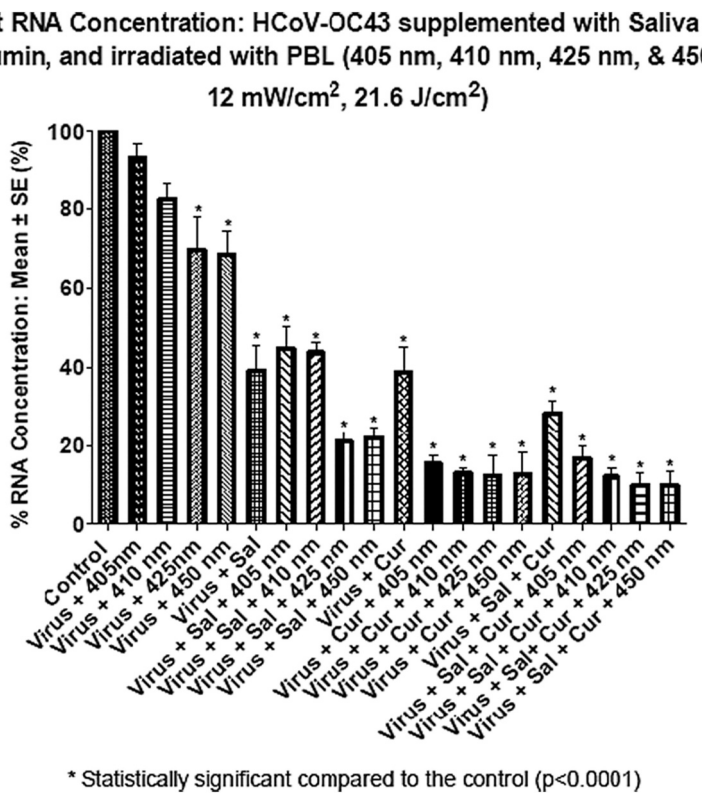


Fig. 7. The Effect of 405, 410, 425 and 450 nm PBL irradiation on saliva and/or curcumin supplemented HCoV-OC43 viral samples. * Indicates statistically significant difference compared to control; there was a statistically significant reduction in RNA concentration at each wavelength tested, more so than PBL irradiation alone, irradiation of saliva supplemented samples, irradiation of curcumin supplemented samples. (sal = saliva; cur = curcumin).

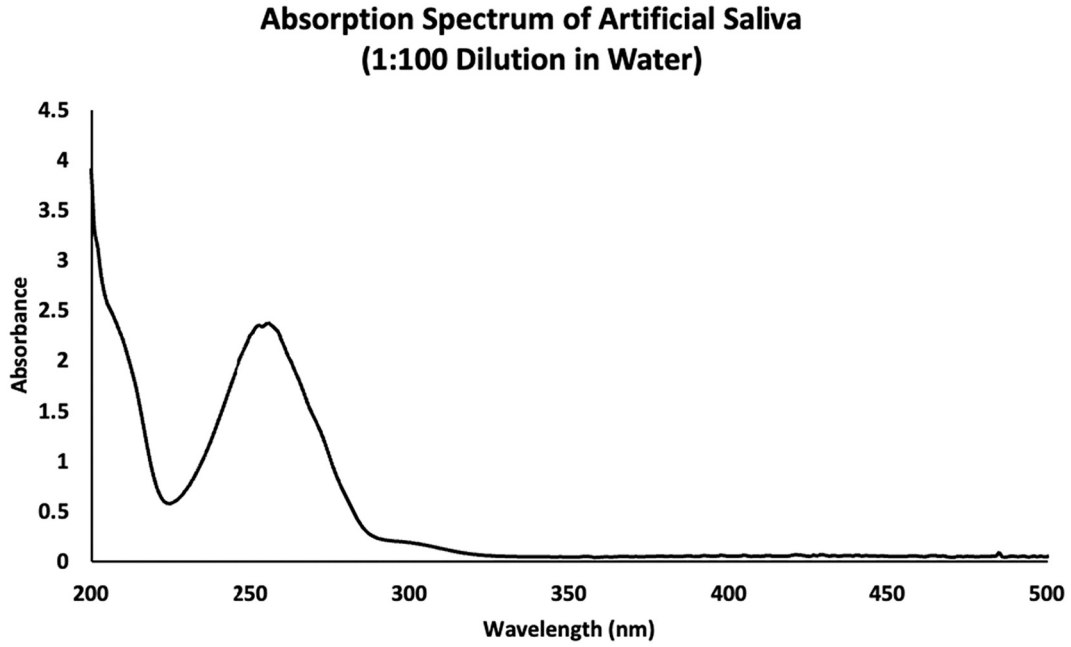


Fig. 8. The absorption spectrum of the artificial saliva used in the experiments. The artificial saliva was obtained from Pickering Laboratories Inc., Mountain View, California.

editing, Analysis and interpretation of data; Violet Bumah: Methodology, Data analysis, Writing-Reviewing and editing; J. Chris Castel: Conceptualization, Review

Declaration of Competing Interest

Drs. Enwemeka and Castel are co-inventors of the Pulsed Blue Light Technology used in this study. Dr. Chris Castel is also the Chief Executive Officer of Carewear Corporation, the company that donated the custom-

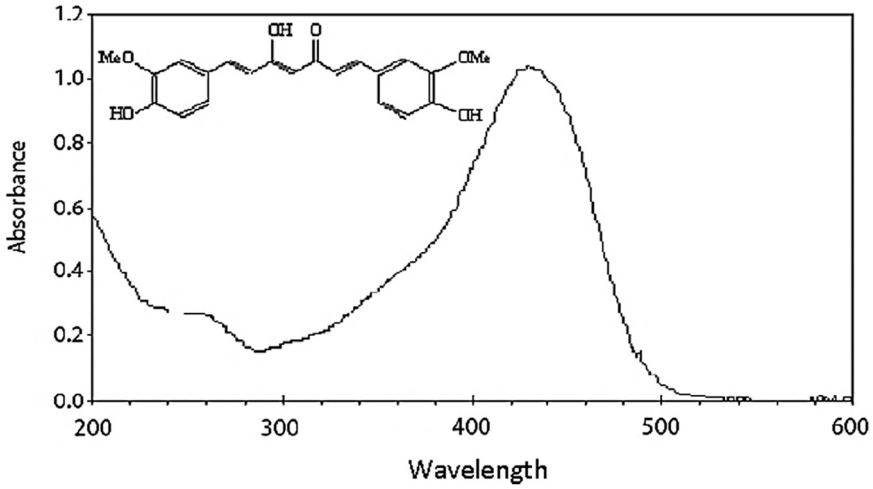


Fig. 9. Standard molecular structure and absorption spectrum of curcumin adapted from R. Waranyoupalin, S. Wongnawa, M. Wongnawa, C. Pakawatchai, P. Panichayupakaranant, P. Sherdshoopongse. (2009) Studies on complex formation between curcumin and Hg (II) ion by spectrophotometric method: A new approach to overcome peak overlap. Cent.eur.j.chem. 7, 388–394. https://doi.org/10.2478/s11532-009-0037-8 (Ref number 80).

made pulsed blue light device used in the study.

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