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Addition of *Camellia sinensis* extract to water to disinfect respiratory viruses accumulated over different surfaces

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ABSTRACT

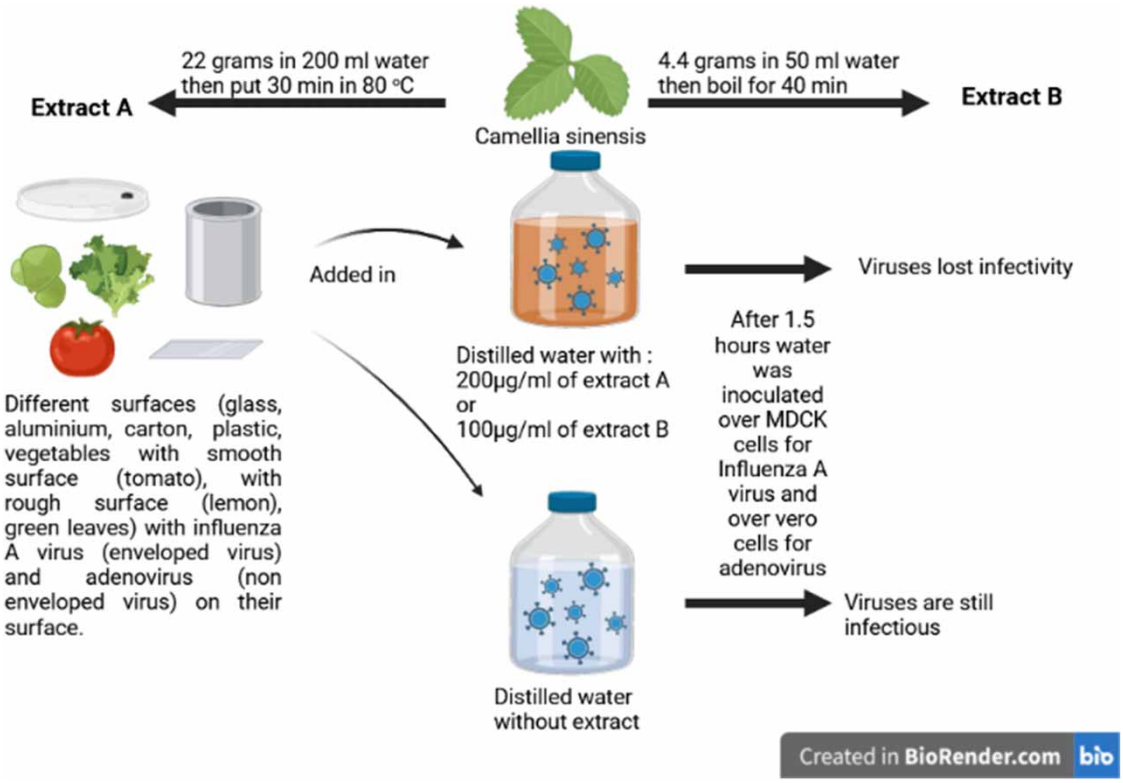
New precautions have become part of our daily life since COVID-19 pandemic such as wearing masks, maintaining distance and disinfecting products bought from markets before using them which is exhausting. We aimed to test the inhibitory effect of *Camellia sinensis* (black tea) water extracts on respiratory viruses and the inhibition of viruses accumulated over different surface types after being soaked in water supplemented with the extracts. Two water extraction methods (extract A: maceration at 80 °C for 30 min and extract B: boiling for 40 min) were applied; extracts were analyzed by high-performance liquid chromatography to detect polyphenolic compounds. Results showed that 200 μg/ml of extract A and 50 μg/ml of extract B in water caused 100% inhibition of influenza A (enveloped virus) virus after 1.5 h and similar results were obtained for adenovirus (non-enveloped virus) but at the same concentration of extract A and at 100 μg/ml of extract B. Different surfaces (aluminum, glass, plastic or carton, vegetables of smooth (tomato) or rough (lemon) surfaces and green leaves) were inoculated with both viruses for 20 min and then soaked in the water supplemented with 200 μg/ml of extract A or 100 μg/ml of extract B for 1.5 h, and this resulted in complete inhibition of both viruses.

Key words: antiviral effect, black tea, *Camellia sinensis* extract, disinfection, polyphenolic compounds

HIGHLIGHTS

- Extracts of natural product represent a treasure of compounds that can work synergistically together better and cheaper than being isolated.
- Water extracts of Camellia sinenses plant have a lot of polyphenolic compounds beside tannins that have direct inhibitory effect on tested viral particles.
- Soaking substances of different surface natures in water containing that extract cause inhibition to accumulated viral particles on those surfaces within 1.5 hours.
- This can be of great benefit during COVID-19 pandemic where accumulated viruses on surfaces bought from markets represent source of infection.

GRAPHICAL ABSTRACT



INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the cause of a global pandemic causing millions of infections and deaths in humans all over the world (Wu *et al.* 2020; Zhu *et al.* 2020). Infection is accompanied by fever, coughing, fatigue, myalgia, headache and diarrhea (Huang *et al.* 2020). However, most people get mild to moderate respiratory illness without any need for further treatment (WHO 2020), whereas others are hospitalized (Grant *et al.* 2020).

The virus spread from the infected person to the uninfected person via contact with infectious viral particles suspended in secretions such as saliva or secretions expelled during coughing, sneezing, or talking to an infected person (Chan *et al.* 2020; Liu *et al.* 2020). People who do not develop symptoms are also the source of infection (WHO 2020).

The SARS-CoV-2 virus can remain infectious for up to 6 days on stainless steel and plastic, 2 days on glass, 7 days on surgical masks, 3 days on banknotes, and on clothes and wood for up to 1 day (Chin *et al.* 2020); so, cleaning the surfaces of different kinds with disinfectants such as ethanol 70%, hypochlorite and hydrogen peroxide is highly recommended (Rutala & Weber 2019).

In our study, we are concerned with disinfecting surfaces of products we buy from groceries, fruits and vegetable shops where infectious viral particles can be accumulated on them via symptomatic or asymptomatic customers and so representing a source of infection and where the above-mentioned disinfectants cannot be used.

Camellia sinensis is a plant known for its antiviral activity against a number of respiratory pathogens including SARS-CoV-2, its activity in many cases comes from its ability to bind to viral particles causing it to lose infectivity (Lyu *et al.* 2005; Yamada *et al.* 2006; Carneiro *et al.* 2016). This was attributed to the presence of a number of polyphenolic compounds (Owuor & Obanda 2007; Teshome 2019) that have the ability to precipitate proteins (Li *et al.* 2010) in addition to tannins that have a high binding ability with protein (Hagerman 2012). The plant has many forms known as white, green, red and black tea. The percentage of tannins is not constant in all of them and black tea was found to contain the highest (Khasnabis *et al.* 2015).

Here, we aimed to test the ability of different water extracts of *C. sinensis* (black tea) to disinfect the respiratory pathogens settling down on different surface types while being soaked in the water supplemented with those extracts.

MATERIALS AND METHODS

Extract preparation, determination of safe doses and determination of polyphenolic chemical composition

Water extraction

C. sinensis powder sold in the market was used (Black tea, Lipton trade). The following two methods were used for extraction:

Method A: 22 g (10 packets) of plant powder was suspended in 200 ml of distilled water and heated for 30 min at 80 °C.

Method B: 4.4 g (two packets) of plant powder was boiled in 50 ml of distilled water for 40 min.

Tea extracts of both methods were filtered through Whatman filter paper and then kept frozen. Total volumes of both extracts were subjected to freeze drying for liquid evaporation and changed it to powder without affecting the composition of the extracts. 10 mg each of the two dried extracts were dissolved in 1 ml of distilled water.

Cytotoxicity of extract

The cell culture safety doses of the extract were determined using the cell morphology technique (Aquino *et al.* 1989). Safety doses were tested on two cell types: MDCK (Madin-Darby canine kidney) cells and Vero (African green monkey kidney cells). A 96-well plate was seeded with cells and incubated overnight. Extracts were inoculated with concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μg/100 μl and observed microscopically for any morphological changes after 24 h incubation at 37 °C in a humidified incubator with 5% CO_2.

Profiling of the polyphenolic compounds and tannin contents of the extracts

Detection of polyphenolic compounds. This was carried out by high-performance liquid chromatography (HPLC): analysis was carried out using an Agilent 1260 series. The separation was carried out using the Eclipse C18 column (4.6 mm × 250 mm; i.d. 5 μm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5–8 min (60% A); 8–12 min (60% A); 12–15 min (82% A); 15–16 min (82% A) and 16–20 (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 5 μl for each of the sample solutions. The column temperature was maintained at 40 °C.

Detection of tannins

A. Ferric chloride test ($FeCl_3$)

A dry weight of 10 mg of each extract was dissolved in 1 ml distilled water, then diluted with a ratio of 1:4 and a few drops of 10% ferric chloride solution were added. Blue or green color indicates the presence of tannins (Evans 1989),

B. Thin layer chromatography (TLC)

A dry weight of 10 mg of each extract was dissolved in 1 ml distilled water and then loaded at starting point of a silica gel TLC plate. At the beginning of the plate, a mixture of solvents consisting of chloroform:methanol:water in a ratio of 6:3:1 was added and left to migrate. A thin layer of 5% ferric chloride was applied over the plate and the presence of tannins was indicated by the formation of a dark brown precipitate at starting point of the plate.

Direct inhibitory effect of the extract on enveloped and non-enveloped viruses

Tested respiratory viruses:

- Non-enveloped virus: Adenovirus type 1, ATCC VR-1 (1.6×10^4 PFU/ml).
- Enveloped virus: influenza A virus (5.2×10^6 PFU/ml): The PR8-H1N1 influenza virus was generated on transfected MDCK cells co-cultured with 293 T-cells with plasmids encoding the eight peptides of the virus as previously reported (Hoffmann *et al.* 2002). This virus is obtained through a material transfer agreement between Dr. Richard Webby at St. Jude Children's Research Hospital and Dr. Mahmoud Bahgat at the NRC, Egypt.

Cells

MDCK cell line was used to propagate influenza A virus. The cells were propagated in the RPMI medium (Lonza, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic–antimycotic mixture (Lonza, USA).

The Vero cell line was used to propagate Adenovirus 1. The cells were propagated in a Dulbecco Modified Eagle Medium (DMEM) (Lonza, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic–antimycotic mixture (Lonza, USA).

Direct inhibitory activity of extracts

The test was carried out according to Schuhmacher *et al.* (2003), in a 24-well plate where Vero cells (for Adenovirus) or MDCK cells (for Influenza virus) were cultivated (10^5 cell/ml) for 1 day at 37 °C. A volume of 1 ml media containing 25, 50, 100, 200, 300 and 400 μg/ml of extracts A and B each at a time, each concentration in a vial mixed with the viral seed of the two tested viruses, Influenza (5.2×10^6 PFU/ml) and adenovirus (1.6×10^4), were prepared and then incubated for 1 h at 37 °C. Ten-fold dilution was made twice to the virus–extract mixture by using media in such a way that a suitable amount of virus but none or minor amount of extract remains and 300 μl was inoculated in each well of 24-well plates after removing growth media over the cells and washing it twice with phosphate buffer saline (PBS). Plates were incubated for 2 h at 37 °C with shaking every 10 min, and then inoculums were removed and 400 μl of media were added. A well was left as cell control and another well was inoculated with the same viral inoculums (same viral count of both viruses were added to 1 ml media and after 1 h incubation at 37 °C ten-fold dilution was made twice and 300 μl was added over cells) without the addition of the extract as positive control then all wells were incubated for 48 h. To test the percentage inhibition of virus as a result of being subjected to the extract, Enzyme-Linked Immunosorbent Assay (ELISA) was made for influenza A virus and adenovirus detection.

ELISA was performed for antigen detection using cell lysates of different treatments. 96-well plates were coated with 50 μg/ml in three replicates of each lysed cell treatment for 2.5 h (carbonate buffer, pH=9.6). Wells were blocked with 150 μl/well blocking buffer (PBS-0.05%-Tween20 (PBST), 5%-FBS) for 2 h then incubated with 50 μl/well of either 1:2,000 dilution of influenza antibody or 1:200 of adenovirus antibody for 1 h. This was followed by the application of 1:1,000 dilution of horse-radish-peroxidase (HRP)-conjugated anti-mouse IgG antibody (Bio-Rad Laboratories, Germany) for 1 h. Wells were then incubated with HRP-specific substrate (0.04% *O*-phenylenediamine, Sigma-Aldrich; Germany; in Citrate buffer +30% H_2O_2). Color development was stopped by adding 50 μl/well 2M H_2SO_4. The optical density (OD) values (absorbance) were recorded using a multi-well plate reader (Tecan, Switzerland) at wavelength λ 492 nm and a 620-nm filter as a reference wavelength. All assay incubations were carried out at 37 °C, wells were washed four times prior to each step during the whole assay with 300 μl per well with PBST to confirm the removal of all contents of the wells before starting the following step.

Calculations: Readings of all wells (cell control, positive control and wells of viruses treated with extract) were obtained as OD readings, first we calculated the mean of the three readings of every treatment, then subtracted the mean of the reading of cell control wells from all other treatments including the positive control then the following equation was applied:

$$\frac{\text{Positive control reading}-\text{Treated virus reading}}{\text{Positive control reading}} \times 100$$

Direct inhibitory effect of extracts in water

A volume of 10 ml of distilled water was supplemented with 200, 300 and 400 μg/ml of extract A for both influenza A and adenovirus type 1 and 50, 100 and 200 μg/ml of extract B for influenza A virus and 100, 200 and 300 μg/ml of extract B for adenovirus type 1. Viruses were added to give final titers of 5.2×10^4 PFU/ml for influenza A virus and 1.6×10^2 PFU/ml for adenovirus type 1. A volume of 10 ml water was directly inoculated with the same viral inoculum without the addition of the extract and considered as a positive control. After 30 min, 1 and 1.5 h incubation at room temperature, 300 μl from each tube were used to inoculate MDCK cells in case of influenza and Vero cells in case of type 1 adenovirus, and the inhibition of both viruses was recorded as mentioned above.

Direct inhibitory effect of extracts on viruses accumulated on different surfaces

Volumes of 20 μl of Influenza virus or adenovirus 1 stocks were inoculated over different surfaces with area 1 cm × 1 cm (glass, plastic, carton and aluminum) and on vegetables of different nature (smooth surface such as tomato, granulated surface such as lemon and leafy plants such as lettuce) and incubated for 20 min. Each piece was put in 10 ml distilled water once supplemented with 200 μg of extract A or100 μg of extract B then incubated for 1.5 h and once in 10 ml distilled water without extracts as a positive control to exclude any adsorption capacity of surfaces. Also, 10 ml of water was directly inoculated with the same viral inoculums without the addition of the extract and considered as positive virus controls. The inhibition of both viruses was recorded as mentioned above.

All previously mentioned experiments were repeated three times and the average was calculated and used to get standard deviation values.

RESULTS AND DISCUSSION

Cytotoxicity test

To exclude any harmful effect of the extract, a cytotoxicity check was done on both Vero and MDCK cells and the results are summarized in Table 1. Table 1 shows that for Vero cells, extract A was completely safe up to 90 μg/100 μl and toxicity starts from 100 μg/100 μl, whereas extract B starts showing toxicity at 90 μg/100 μl.

For MDCK cells, extract A was safe up to 90 μg/100 μl, and extract B was safe up to 50 μg/100 μl and start showing toxicity from 60 μg/100 μl.

Detection of polyphenolic compounds and tannins contents of the extracts

Polyphenolic compounds

Profiling of the polyphenolic compounds by HPLC in both extracts is summarized in Table 2. Results showed that the concentration of all tested compounds except the daidzein was higher in extract B than in extract A and this might be due to the effect of boiling for 40 min which causes more cell destruction and so more concentrations of all compounds.

Table 1 | Evaluating cytotoxicity of different concentrations of *Camellia sinensis* water extracts on Vero and MDCK cells

Conc. (μg/100 μl)	10	20	30	40	50	60	70	80	90	100
Extract A/Vero cells	–	–	–	–	–	–	–	–	–	+1
Extract B/Vero cells	–	–	–	–	–	–	–	–	+1	+2
Extract A/MDCK cells	–	–	–	–	–	–	–	–	–	+1
Extract B/MDCK cells	–	–	–	–	–	+1	+1	+2	+3	+4

'–' indicates safe dose, '+1' indicates 25% of dead cells '+2' indicates 50% of dead cells, '+3' indicates 75% of dead cells, '+4' indicates 100% of dead cells.

Table 2 | Polyphenolic compounds present in the methanolic extract of *Camellia sinensis* as detected by HPLC

		Concentration (μg/ml)	
Compound	Standards (μg/ml)	Extract A	Extract B
Gallic acid	8.4	231.79	1,006.57
Chlorogenic acid	14	26.72	166.98
Total catechins	33.75	20.92	38.71
Methyl gallate	5.1	3.47	9.21
Caffeic acid	9	ND	55.35
Syringic acid	8.6	0.42	ND
Pyro catechol	14.6	4.16	20.42
Rutin	30.5	20.36	46.96
Ellagic acid	17.15	23.66	64.71
Coumaric acid	6.6	6.21	12.93
Vanillin	6.45	0.41	0.22
Ferulic acid	6.2	13.78	19.35
Naringenin	7.5	11.92	90.09
Daidzein	10	5.90	1.08
Querectin	6.4	ND	0.13
Cinnamic acid	2.9	ND	0.84

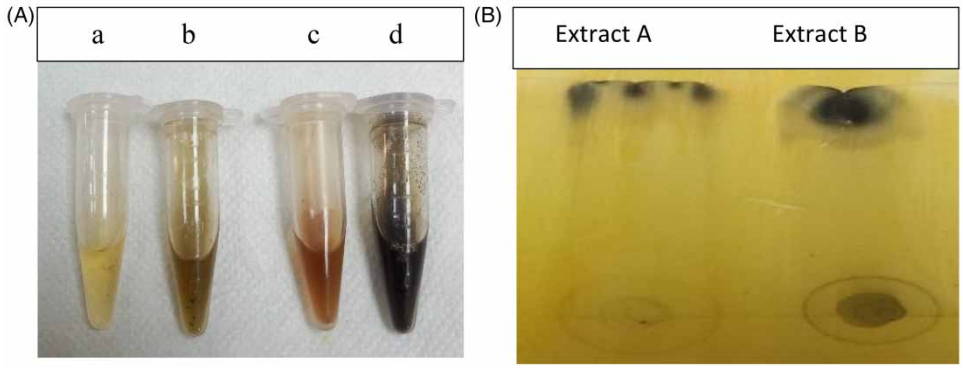


Figure 1 | Detection of tannins. (A) Addition of $FeCl_3$ to the diluted extract, where (a) indicates extract A without $FeCl_3$, (b) indicates extract A with $FeCl_3$ that resulted in the presence of light green precipitate, (c) indicates extract B without $FeCl_3$, (d) indicates extract B with $FeCl_3$ that resulted in dark green precipitate. (B) TLC plate where extract A caused dark precipitate at the starting point, whereas extract B caused more dense dark color at the same point. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wh.2022.072.

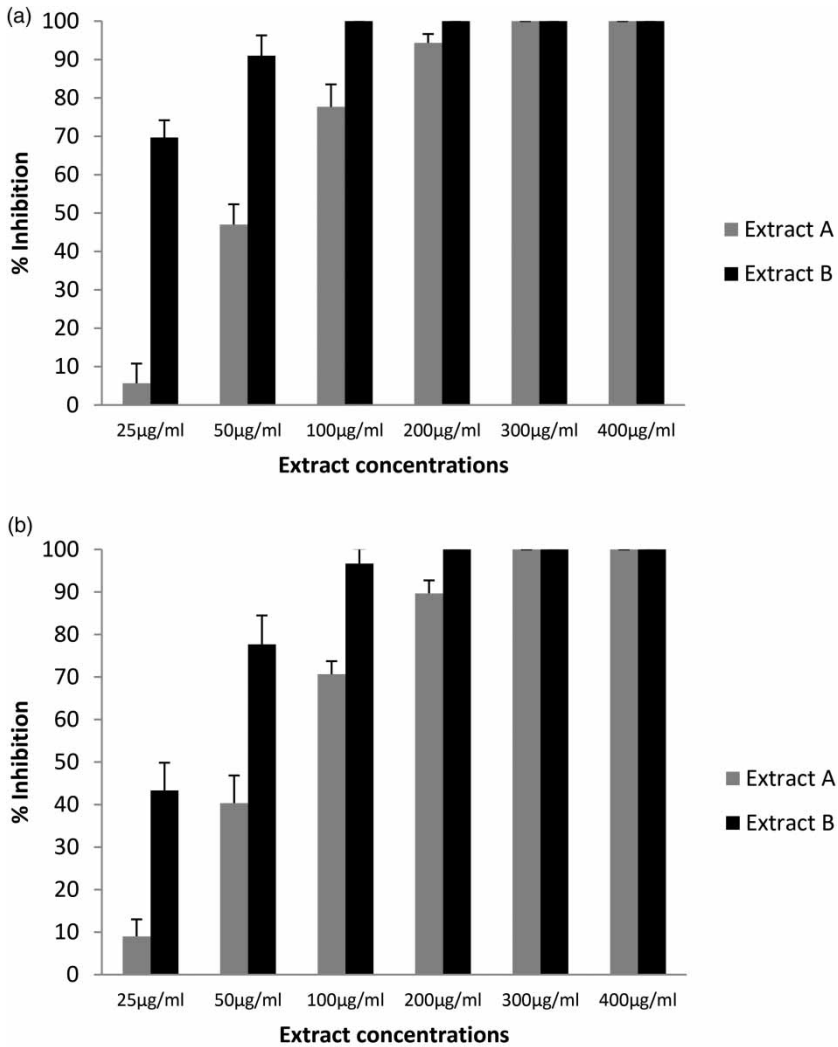


Figure 2 | Direct inhibitory effect of extract A and extract B on (a) influenza A virus and (b) adenovirus type 1 after 1 h of incubation.

Tannins

Tannins are a group of compounds found in green plants and are known to bind irreversibly to proteins, and they are found in a high percentage in *C. sinensis* plant, especially, black tea and it is responsible for its bitter taste (Khasnabis *et al.* 2015).

Using the $FeCl_3$ method results in Figure 1(a) showed the appearance of green precipitate (ppt) which was darker in extract B than in extract A. Using TLC results in Figure 1(b) showed the appearance of a dark spot at the starting point of TLC plate that was also darker in extract B. This also might be due to the extraction method applied to extract B that causes more cell destruction and thus allow more contents to be released.

Direct inhibitory activity of both extracts

On testing the direct inhibitory effect of extracts A and B on influenza A virus, results in Figure 2(a) showed that extract A was able to cause 100% viral inhibition at 300 μg/ml while extract B gave the same percentage of inhibition at 100 μg/ml. On the other hand, on applying the same test on adenovirus type 1, results in Figure 2(b) showed that extract A was able to cause 100% viral inhibition at 300 μg/ml while extract B gave the same percentage of inhibition at 200 μg/ml.

The direct inhibitory effect of *C. sinensis* extract was previously reported on influenza A virus (Yamada *et al.* 2006) and on adenovirus (Karimi *et al.* 2016). Extract B always showed higher percentage of inhibition than extract A at all used concentrations. This is due to the higher amount of polyphenolic compounds and tannins in it. Reports showed the ability of those compounds especially tannins to bind with protein and so cause the virus to lose its ability to infect cells.

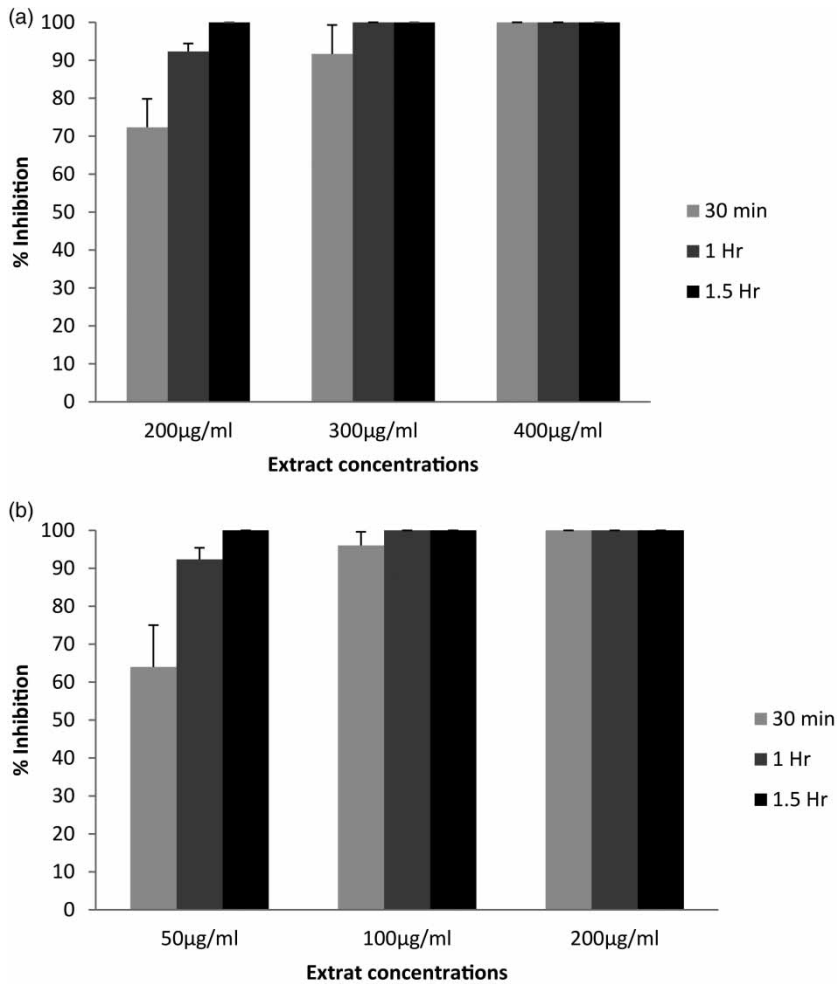


Figure 3 | Inhibitory effect of (a) extract A and (b) extract B on influenza A virus in water at different time intervals.

Direct inhibitory effects of extracts in water

Extracts A and B were applied to 10 ml distilled water at different concentrations starting from the one causing more than 95% viral inhibition in the previous experiment. For influenza A virus: extract A was applied at 200, 300 and 400 μg/ml and extract B at 50, 100 and 200 μg/ml. For adenovirus type 1, extract A was applied at 200, 300 and 400 μg/ml and extract B at 100, 200 and 300 μg/ml. The virus was inoculated over cells after 30 min, 1 and 1.5 h.

Results showed that a 100% viral inhibition was obtained when applying 200 μg/ml of extract A and 50 μg/ml of extract B for 1.5 h to water containing influenza A virus (Figure 3(a) and 3(b)) and the same percentage of inhibition was obtained with the addition of 200 μg/ml of extract A and 100 μg/ml of extract B for 1.5 h to water containing adenovirus type 1 (Figure 4(a) and 4(b)).

Direct inhibitory effects of extracts on viruses accumulated on different surfaces

On applying both viruses one at a time over the surface of glass, aluminum, plastic, carton, lemon, tomato and green leaves showed no adsorption capacities over various surfaces as no percentage of inhibition appeared except for lemon and tomato that were able to make a 100% viral inhibition to influenza virus (Figure 5(a)) and 34 and 13% inhibition from lemon and tomato, respectively, to adenovirus type 1 (Figure 5(b)). On the other hand, applying both viruses on different surfaces, then soaking them in distilled water supplemented with 200 μg of extract A or 100 μg extract B and then incubating for 1.5 h resulted in complete inactivation of both viruses compared to the positive control.

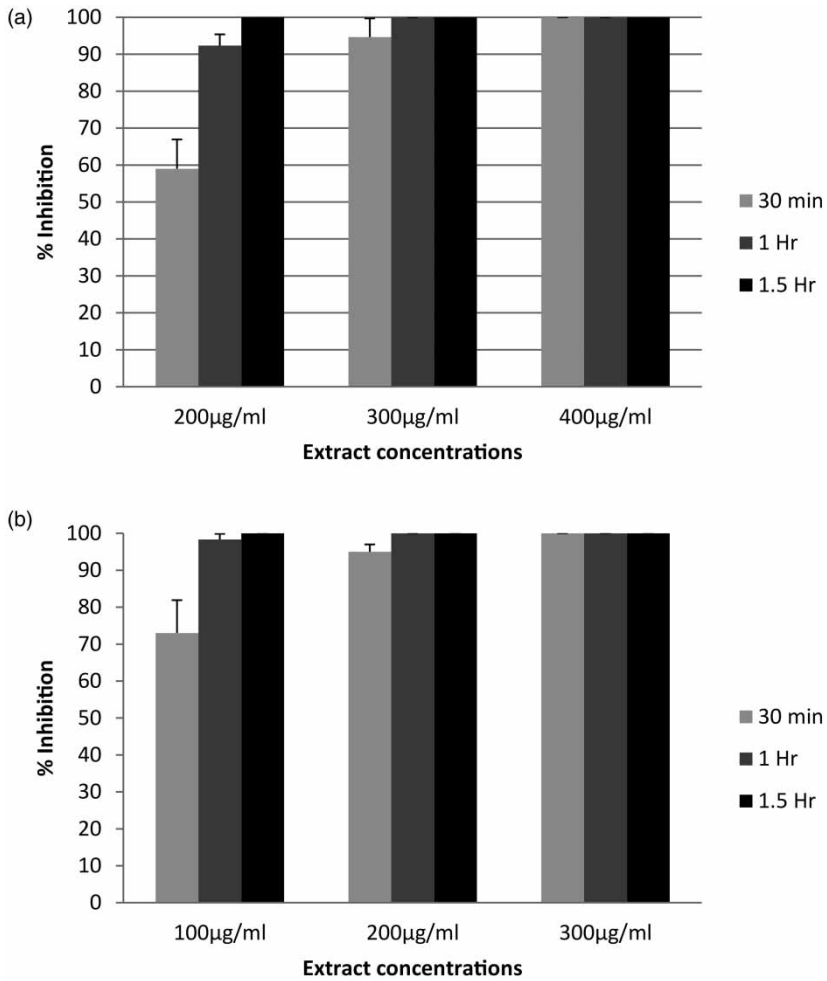


Figure 4 | Inhibitory effect of (a) extract A and (b) extract B on adenovirus type 1 virus in water at different time intervals.

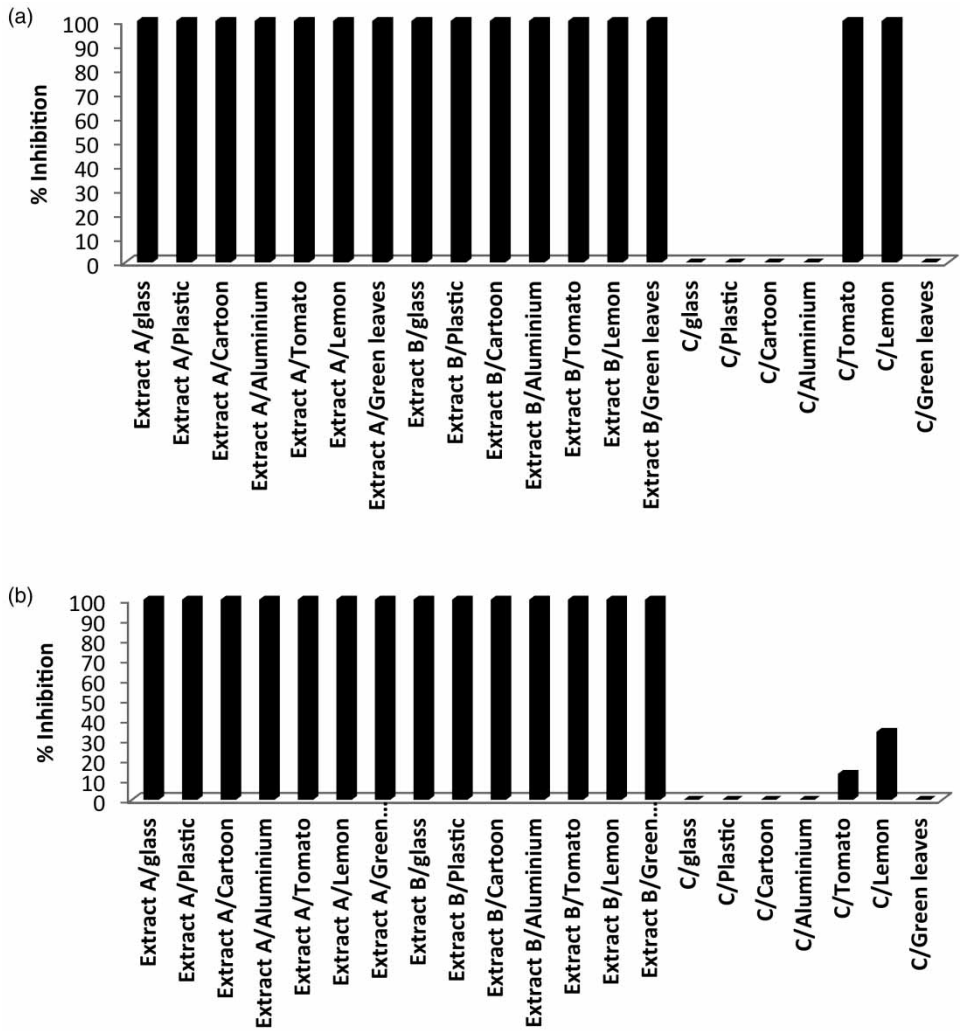


Figure 5 | Inhibitory effect on (a) influenza A virus and (b) adenovirus virus applied over different surfaces and then soaked in water supplemented with the extracts for 1 h.

The percentage of reduction caused by the lemon surface might be due to the presence of citric acid which was reported to show inhibitory effect on influenza virus and other viruses as well (Oxford *et al.* 1971; Balta *et al.* 2020), but for tomato, more studies need to be done to test its antiviral effect. Viral inhibition might also be caused by a difference in pH caused by lemon or tomato which also needs to be well studied.

CONCLUSION

Addition of 20 mg/L of extract A or 10 mg/L of extract B to water and using the extract–water mixture to soak materials of different types for 1.5 h helps in getting rid of accumulated viral particles over their surfaces. Being a water extract of a natural plant, which people normally drink increases their safety particularly to disinfect food stuff.

FUNDING

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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