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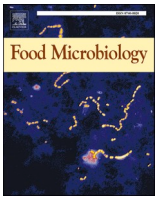

Edible alginate-based films with anti-SARS-CoV-2 activity

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ABSTRACT

The viability of SARS-CoV-2 on food surfaces and its propagation through the food chain has been discussed by several stakeholders, as it may represent a serious public health problem, bringing new challenges to the food system. This work shows for the first time that edible films can be used against SARS-CoV-2. Sodium alginate-based films containing gallic acid, geraniol, and green tea extract were evaluated in terms of their antiviral activity against SARS-CoV-2. The results showed that all these films have strong *in vitro* antiviral activity against this virus. However, a higher concentration of the active compound (1.25%) is needed for the film containing gallic acid to achieve similar results to those obtained for lower concentrations of geraniol and green tea extract (0.313%). Furthermore, critical concentrations of the active compounds in the films were used to evaluate their stability during storage. Results showed that gallic acid-loaded films lose their activity from the second week of storage, while films with geraniol and green tea extract only show a drop in activity after four weeks. These results highlight the possibility of using edible films and coatings as antiviral materials on food surfaces or food contact materials, which may help to reduce the spreading of viruses through the food chain.

1. Introduction

The use of films and coatings produced from bio-based materials, such as proteins, polysaccharides and lipids, is an environmentally friendly strategy that offers substantial advantages in increasing the shelf life and improving the quality and food safety of fruits and vegetables (Flores-López et al., 2016; Guimarães et al., 2018). They can act as a barrier against mass diffusion (moisture and gases) and retard spoilage and surface growth of bacteria, yeasts and molds on a wide range of products. They can also be used in the functionalization of surfaces, such as food packaging materials (e.g., flexible plastic films) (Costa et al., 2021). The incorporation of active and functional compounds in edible films and coatings to develop active materials is an interesting strategy that promotes food safety, extends the shelf life of food, and inhibits the growth of unwanted microorganisms and pathogens (Costa et al., 2018; Guimarães et al., 2020).

There is much controversy regarding the viability of SARS-CoV-2, the virus responsible for the COVID-19 pandemics, on food surfaces and its ability to spread through the food chain. Recently, Jung et al. (2023) showed that SARS-CoV-2 is maintained viable for days on food surfaces, but that viability depends on the type of food and storage temperature. For example, they showed that infectious SARS-CoV-2 was detected for up to 10 days at 4 °C. Therefore, investigating the use of materials that can minimize SARS-CoV-2 presence in food and food packaging is of great interest (Ceylan et al., 2020; Ezzatpanah et al., 2022; Yekta et al., 2021). Thus, the use of antiviral compounds in packaging materials, such as polyethylene (Mizielińska et al., 2021; Ordon et al., 2021) and polyethylene terephthalate (Zhou et al., 2021), has been proposed and their ability to inactivate SARS-CoV-2 has been tested.

The antimicrobial capacity of various natural compounds, such as organic acids, enzymes, plant-derived compounds and their by-products, such as essential oils and polyphenols, has already been proven (Saklani et al., 2019). However, information on the use of antiviral compounds in edible films and coatings is still very limited. Recently, Fabra et al. (2018) reported the antiviral capacity of alginate films containing phenolic extracts, such as green tea extract (GTE) and grape seed extract against murine norovirus (MNV), the most widely used human norovirus surrogate, and hepatitis A virus (HAV). The same

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research group also described the antiviral effect of carrageenans (κ−, ι− and λ−) and GTE incorporated in film-forming dispersions (FFD) as a coating on blueberries and raspberries, at room temperature (25 °C) and refrigerated temperature (10 °C), against MNV and HAV (Falcó et al., 2019). In 2020, Amankwaah et al. assessed the effectiveness of chitosan-based films enriched with GTE against MNV. Another interesting work by Moreno et al. (2020) compared the antiviral potential of three different polysaccharide-based matrices (agar, alginate and agar/alginate mixture) loaded with a polyphenol extract from *Larrea nigra* plant. Finally, in a study carried out by Sharif et al. (2021), the use of allyl isothiocyanate (AITC), a volatile and aliphatic sulfur-containing compound with recognized antimicrobial activity, was evaluated in blueberries as a potential antiviral compound against MNV.

Some works have also shown that compounds of natural origin have promising antiviral activity against SARS-CoV-2 (Liu et al., 2021; Senthil Kumar et al., 2020); however, there are still no studies on the effectiveness of coatings and films based on those food-grade materials against this virus. This work shows, for the first time, the possibility of using edible films of natural origin with antiviral capacity as active materials against SARS-CoV-2.

2. Materials and methods

2.1. Film-forming solutions

Film-forming solutions were prepared by dissolving 1.0% (w/v) sodium alginate (CEAMTEX® 1691, Ceamsa, Spain) in distilled water. Sucrose ester (Ryoto® P1570, Mitsubishi Chemical, USA) (0.5%, w/v) was added to the solution as a surfactant and as an emulsifier to assist in the dissolution of some active compounds tested. The solution was left at room temperature (20 °C) for 4 h, under agitation using a magnetic stirrer, and then heated at 80 °C for 30 min. The active compounds gallic acid (GA) (Penta International Corporation, USA), green tea extract (GTE) (Teavigo®; epigallocatechin gallate content 95.3%) and geraniol (G) (W250708, Sigma-Aldrich, USA) were then added and left stirring for 16 h at room temperature. The concentrations tested for each compound were defined based on preliminary studies and are presented in Table 1. All the materials used are approved for food applications by the European Union regulation, as listed in Regulation (EC) 1333/2008 (sodium alginate and sucrose ester), Regulation (EC) No 1334/2008 (GA and G) and Regulation (EC) No 1925/2006 (GTE).

2.2. Film production

A volume of 1 mL of each solution was cast onto slides (75 × 25 mm^2 polystyrene walls with soda-lime glass bottom) and placed in an oven at 35 °C for 4 h. They were subsequently stored at room temperature in the absence of light until further tests.

2.3. Antiviral tests

Dose-response analyses of samples consisting of slides coated with different concentrations of active ingredients versus recovered infectious virus particles were performed to determine critical doses corresponding to an excellent antiviral effect (3-log reduction) (ISO, 2019). To achieve that, samples were exposed to 200 μL of SARS-CoV-2 suspension (corresponding to 1.6 × 10^5 PFU) and incubated at room temperature for 60 min, before being washed with 10 mL of washing solution (tryptic soy broth with 0.07% lecithin and 0.5% polysorbate 80). Subsequently, 10-fold serial dilutions were prepared by transferring 200 μL of the washed solution to a well containing 1.8 mL of maintenance medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% heat-inactivated fetal bovine serum (HI-FBS), 50 U/mL penicillin, 50 μg/mL streptomycin and 2 mM glutamine) and repeated twice, yielding 10^{-1}, 10^{-2} and 10^{-3} dilutions.

Table 1
Concentrations of active compounds in film-forming solutions.

Sample	Gallic acid (GA)	Green tea extract (GTE)	Geraniol (G)
1	0.156% (GA1)	0.156% (GTE1)	0.156% (G1)
2	0.313% (GA2)	0.313% (GTE2)	0.313% (G2)
3	0.625% (GA3)	0.625% (GTE3)	0.625% (G3)
4	1.25% (GA4)	1.25% (GTE4)	1.25% (G4)
5	2.50% (GA5)	2.50% (GTE5)	2.50% (G5)

After every antiviral test, the recovered infectious virus particles from each tested sample were quantified by plaque assay as previously described (Tomás et al., 2022). Briefly, Vero cell CCL-81 (ATCC) monolayers were inoculated in duplicates with 500 μL of 10-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of the washed-out virus. Cells were incubated in a CO_2 incubator at 37 °C, for 1 h, with gentle rocking every 15 min. Subsequently, the virus inoculum was removed, and 2 mL of overlay medium (maintenance medium with 1.25% (w/v) carboxymethylcellulose) was added to the cell monolayer. The overlay medium was removed after 4 days of incubation, and cells were fixed with 4% formaldehyde in PBS and stained with 0.05% (w/v) toluidine blue to visualize viral plaques. The viral titer was calculated as plaque-forming units per milliliter (PFU/mL), according to:

$$\frac{PFU}{mL} = Cp \times \frac{1}{d} \times \frac{1}{v} \quad (1)$$

where C_p is the average number of plaques counted in all triplicates, d is the dilution at which the plate count was made, and v is the volume of washed-out virus used to perform the plaque assay. Under these experimental conditions, the detection limit of the plaque assay was 1 PFU at the lowest dilution tested (10^{-1}).

The antiviral activity (M_v) of each sample was calculated from the relation between the active virus recovery from the active film samples and the recovery from control samples (alginate-based film samples) after 60 min of contact time, according to:

$$M_v = log(V_a) - log(V_b) \quad (2)$$

where *log* (V_a) is the common logarithm average of 3 infectivity titer values 60 min after inoculation of the control sample (alginate sample), and *log* (V_b) is the common logarithm average of 3 infectivity titer values after 60 min of contact time with the film.

At the end, the percentage of viral activity reduction ($R_{\%}$) was calculated as:

$$R_{\%} = \left(1 - \frac{1}{10^{M_v}}\right) \times 100 \quad (3)$$

2.4. Antiviral activity of films during storage

In order to evaluate the loss of antiviral activity of films during storage, antiviral tests were performed in films right after production (week 0) and after 2, 3 and 4 weeks of storage at room temperature (week 2, week 3 and week 4). For these tests, only films developed with 1% (w/v) of sodium alginate, 0.5% (w/v) of sucrose ester and the active compounds at the selected critical concentrations were tested.

3. Results and discussion

The first step was the evaluation of the antiviral activity (M_v) of the films made of 1% (w/v) sodium alginate and 1% (w/v) sodium alginate combined with 0.5% (w/v) of sucrose ester. These films were tested to evaluate the antiviral efficacy of the base component of the films (alginate or alginate with sucrose ester). M_v values were used as control to obtain the real antiviral activity of the active compounds added to the films (GA, GTE and G). Results showed that alginate-based films (without any other compound) present some antiviral activity against

SARS-CoV-2 (M_v = 0.68 ± 0.31). However, this antiviral activity is boosted upon combining alginate with sucrose ester (M_v = 2.81 ± 0.32), which leads already to a 2-log reduction of viral activity (99.80% ± 0.15%). This may be explained by the ability of sucrose ester to destabilize the viral envelope, as a consequence of its tensioactive properties. This behavior has already been proposed to explain the sucrose ester activity against bacteria and fungi (Teng et al., 2021), but it had never been demonstrated before against a virus.

3.1. Antiviral activity of gallic acid (GA)

Gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally occurring aromatic polyphenolic acid mostly found in some fruits and plants. It has been reported as a strong natural antioxidant against different reactive oxygen species (Uddin et al., 2022). In addition, GA is reported to affect multiple pharmacological and biochemical pathways, exerting different biological activities, including anti-inflammatory, anti-diabetic, cardioprotective, anticancer and hepatoprotective activity (Uddin et al., 2022). GA is approved for food applications and can be used in foods as a flavoring agent, being, therefore, a good candidate to be used as an antiviral compound against SARS-CoV-2 on food surfaces or food contact materials.

Our dose-response analyses show increased antiviral activity with increasing GA concentrations (Fig. 1A). Furthermore, films loaded with 1.25% and 2.5% (w/v) of GA (GA4 and GA5, respectively) presented a higher antiviral effect, when compared with films made only of sodium alginate and sucrose ester. These concentrations were even sufficient to reach the method's viral detection limit (i.e., no active viral particles were recovered after the contact time tested), showing that 1.25% (w/v) of GA should be selected as the critical concentration able to produce, at least, a 3-log reduction of viral activity.

These results are in agreement with other works that have shown that gallic acid presents a broad-spectrum antiviral activity against other viruses. For instance, Govea-Salas et al. (2016) showed that gallic acid is effective against hepatitis C virus (HCV) through its antioxidant effect. The compound decreases the rate of viral protein production, leading to a decrease in cellular oxidative stress, which is unfavorable to the virus. Furthermore, Kratz et al. (2008) demonstrated that GA has antiviral activity against herpes simplex virus (HSV) by inhibiting the binding and penetration of the virus into cells, preventing cell-cell viral spread. Until now, there were no *in vitro* or *in vivo* studies evaluating GA activity against SARS-CoV-2. However, Umar et al. (2021), in a molecular docking assay, proposed the potential inhibitory action of sixteen gallic acid derivatives that can bind to five key proteins of SARS-CoV-2: nsp3 (papain-like protease), nsp5 (major protease), nsp12 (RNA-dependent RNA polymerase), nsp13 (helicase) and nsp14 (nidoviral uridylate-specific endoribonuclease).

3.2. Antiviral activity of green tea extract (GTE)

Green tea extract is a polyphenol extract obtained from the tea plant (*Camellia sinensis* L.), of the Theaceae family. The main polyphenols present in GTE are catechins that include (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG). EGCG, reported to be the main catechin in green tea (~50%), presents antioxidant, anticarcinogenic, anti-inflammatory and antimicrobial (bactericidal and virucidal) properties against a wide range of food-borne pathogens (Perumalla and Hettiarachchy, 2011). Their activity against viruses has also been shown against SARS-CoV-2 elsewhere (Kicker et al., 2022; Nishimura et al., 2021).

The GTE antiviral activity against SARS-CoV-2 was also assessed in this work (Fig. 1B). This extract presented an antiviral effect above 3 at 0.156% (w/v) (GTE1), guaranteeing a 3-log reduction in viral activity at 0.313% (w/v) (GTE2), and showing higher antiviral activity than gallic acid for low concentrations.

The mechanism of action of the green tea extract against SARS-CoV-2 was explained by Liu et al. (2021). They showed that EGCG can inhibit SARS-CoV-2 3CL protease, an enzyme important for the infection and replication of the coronavirus in the host, and that EGCG blocks the viral binding of SARS-CoV-2 host cells to the ACE2 receptor, thus acting as an antiviral compound to the coronavirus. The effectiveness of GTE against viruses, more specifically EGCG, has been widely reported by several authors. For example, Xu et al. (2008) proved that EGCG reduces not only the expression of hepatitis B virus-specific antigens, but also the levels of extracellular DNA, intracellular replicative intermediates, and viral covalently closed circular DNA (cccDNA). Similarly, Ciesek et al. (2011) demonstrated the antiviral efficacy of EGCG against HCV, inhibiting the entry of all virus genotypes into primary human hepatoma and hepatocyte cell lines, preventing it from binding to target cells. Furthermore, similarly to the effect of EGCG on HCV, Isaacs et al. (2008) demonstrated that this compound is effective against HSV by directly preventing the binding of the extracellular viral particle to the target cell receptors.

The use of GTE as an antiviral in edible coatings has been previously studied by Fabra et al. (2018). They showed the antiviral capacity of alginate films loaded with GTE against MNV and HAV. Additionally, Falcó et al. (2019) described the antiviral effect of carrageenan (κ−, ι− and λ−) and GTE incorporated in film-forming dispersions (FFD) in blueberries and raspberries, at room temperature (25 °C) and refrigerated temperature (10 °C), against MNV and HAV.

3.3. Antiviral activity of geraniol (G)

Geraniol is a monoterpenic alcohol found in more than 250 essential oils, such as *Monarda fistulosa* (wild bergamot), ninde, rose and

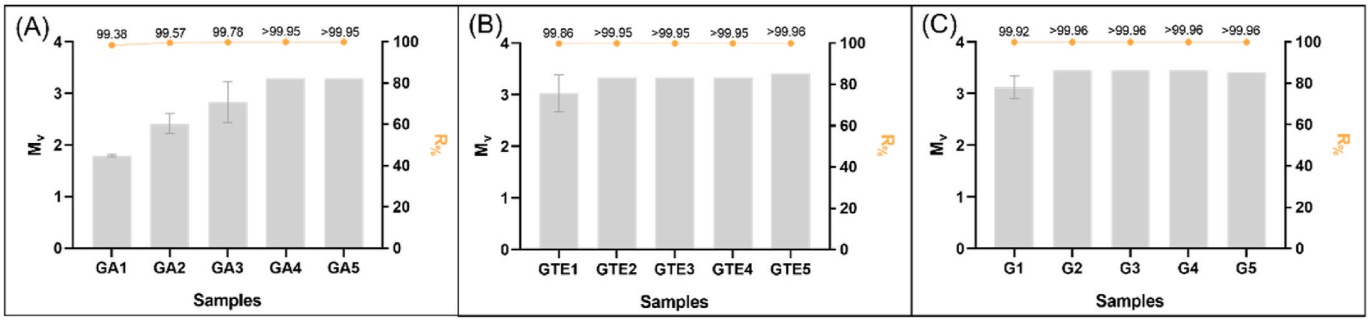

Fig. 1. Dose-response behavior of antiviral activity (M_v) and percentage of SARS-CoV-2 activity reduction ($R_{\%}$) using sodium-alginate-based films with different concentrations of: (A) gallic acid (GA), (B) green tea extract (GTE) and (C) geraniol (G). Bars represent the M_v values after a contact time of 60 min. Dots and line represent the corresponding percentages of viral inactivation, being data presented as mean ± standard deviation. The symbol ">" is used when no infectious virus particles were recovered, being assumed a value above the detection limit.

palmarosa oils (Mączka et al., 2020). Geraniol has a great number of biological activities, such as antioxidant, anti-inflammatory, antimicrobial and plant insect repellent (Mączka et al., 2020). Its antiviral activity against SARS-CoV-2 was proposed by Kulkarni et al. (2020) in a molecular docking study, where the authors showed its potential to inhibit the viral spike protein.

In the present work, the activity against SARS-CoV-2 of geraniol was experimentally demonstrated (Fig. 1C). Geraniol presented a behaviour similar to GTE, showing an antiviral critical concentration of 0.313% (w/v) (G2), which guarantees an M_v greater than 3 and a percentage of reduction above 99.96%.

The antiviral activity of geraniol was also shown by Senthil Kumar et al. (2020). They found that geraniol was able to reduce the levels of angiotensin-converting enzyme 2 (ACE2), the key SARS-CoV-2 host cell receptor, from 18.0 ng/mL (control) to 10.44 ng/mL. Hassan et al. (2018) also showed that geraniol had pronounced inhibitory properties against HSV replication. They showed that geraniol establishes an interaction with the virus protease and promotes its inhibition. When geraniol binds to this enzyme, it competes with the substrate, acting as a competitive inhibitor. Other studies revealed that geraniol had no antiviral effect against Influenza A virus and respiratory syncytial virus (Mileva et al., 2015). This absence of antiviral capacity was also shown by Mizielińska et al. (2021), who studied the ability of geraniol as an external coating in packaging materials against the phage phi 6, chosen as a SARS-CoV-2 surrogate. They showed that geraniol at 0.0125% (w/w) in the coating does not significantly affect phi 6.

3.4. Antiviral activity of films upon storage

The effective use of active films and coatings on foods or packaging requires that they maintain their properties during food storage and transportation. Therefore, the loss of antiviral activity of the films was evaluated over 4 weeks (Fig. 2).

In our assessment, the antiviral activity of films loaded with 1.25% (w/v) of GA (GA4) started to decrease slightly but continuously from week 2, leading to a drop on $R_{\%}$ from above 99.95% to 99.72%. These results indicate that, although active, GA film loses its effectiveness over time, which translates into a limitation in using this film to achieve a 3-log reduction of viral activity for more than two weeks after application. For GTE, a concentration of 0.313% (GTE2) was used for testing. For this film, an antiviral activity that guarantees at least a 3-log reduction of viral activity ($R_{\%} > 99.95\%$) remained over 3 weeks of storage. Only after the fourth week of storage a sharp decrease in activity was noticed, with $R_{\%}$ dropping to 99.79% ± 0.19%, even though still guarantees a 2-log reduction of viral activity. Regarding geraniol, for the selected formulation (G2), its antiviral activity slowly decreased over time, from $R_{\%} > 99.96\%$ at week 0 to $R_{\%} = 99.93\% \pm 0.06\%$ at weeks 2 and 3. Still, a 2- to 3-log reduction of viral activity is guaranteed even after four weeks of analysis (99.85% ± 0.15%). These results show that geraniol presents a slightly more stable antiviral activity, when compared to GA or GTE. This may be justified by the oxidation process that may become more significant for these last two compounds, influencing their antiviral activity. It was also observed that after 4 weeks of storage, films loaded with GA and GTE showed some changes in color (data not shown), which may be associated with an oxidation process.

This slight decrease in activity can be considered expectable for natural compounds, due to their susceptibility to light, oxygen and temperature. However, the magnitude of the decrease experimentally recorded in our study was considerably lower than what was reported by other authors. For example, Randazzo et al. (2017) studied the inhibitory effect of green tea extract on MNV and hepatovirus A (HVA) and its effectiveness in surface disinfection. They determined the antiviral activity of 5 mg/mL of GTE after 2 h at 37 °C, which corresponded to 1.98- and 2.42-log reduction for HVA and MNV, respectively.

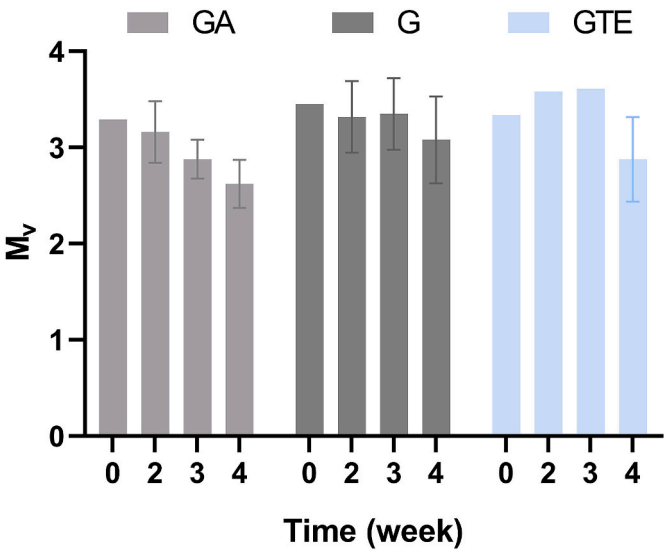

Fig. 2. Changes on the antiviral activity (M_v) against SARS-CoV-2 of sodium alginate-based films with 1.25% (w/v) of gallic acid (GA), 0.313% (w/v) of green tea extract (GTE) or 0.313% (w/v) of geraniol (G), over four weeks of storage.

4. Conclusion

Edible coatings and films have been used for years as carriers of active compounds for different purposes (e.g., antimicrobial, antioxidant), but their use as a carrier of antiviral compounds is less explored. Their application on food surfaces or packaging materials helps to improve food shelf life and packaging properties, also indicating the potential to prevent the propagation of different diseases.

For the first time, this work shows that active edible films can also be used with high effectiveness for inactivating SARS-CoV-2, expectedly acting as a barrier to COVID-19 spreading. All tested compounds (GA, GTE and G) showed very good antiviral activity, with GTE and G presenting a lower critical concentration and higher stability during storage than GA. Both GTE and G-based films show a 3-log reduction of SARS-CoV-2 activity with a concentration as low as 0.313% in the film-forming solution. These results open the possibility of using edible films and coatings as antiviral materials on food surfaces or food contact materials, which will help to reduce the spreading of viruses through the food chain.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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