Molecular Therapy
Original Article

Debulking influenza and herpes simplex virus strains by a wide-spectrum anti-viral protein formulated in clinical grade chewing gum

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Lack of Herpes Simplex Virus (HSV) vaccine, low vaccination rates of Influenza viruses, waning immunity and viral transmission after vaccination underscore the need to reduce viral loads at their transmission sites. Oral virus transmission is several orders of magnitude higher than nasal transmission. Therefore, in this study, we evaluated neutralization of viruses using a natural viral trap protein (FRIL) formulated in clinical-grade chewing gum. FRIL is highly stable in the lablab bean powder (683 days) and in chewing gum (790 days), and fully functional (794 days) when stored at ambient temperature. They passed the bioburden test with no aerobic bacteria, yeasts/molds, with minimal moisture content (1.28–5.9%). Bean gum extracts trapped HSV-1/HSV-2 75–94% in a dose-dependent manner through virus self-aggregation. Mastication simulator released >50% release of FRIL within 15 min of chewing the bean gum. In plaque reduction assays, >95% neutralization of H1N1 and H3N2 required ~40 mg/mL, HSV-1 160 mg/mL, and HSV-2 74 mg/mL of bean gum for 1,000 copies/mL virus particles. Therefore, a 2000 mg bean gum tablet has more than adequate potency for clinical evaluation and is safe with no detectable levels of glycosides. These observations augur well for evaluating bean gum in human clinical studies to minimize virus infection/transmission.

INTRODUCTION

In today's tightly connected world, infectious disease is becoming a more severe and frequent threat due to several factors, including climate changes, population density, increased international mobility, and rapid urbanization in low-income countries with poor adoption of public health measures.[1,2] Of recent concern is the coronavirus pandemic, in which severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) led to ~7 million documented deaths worldwide.[3] In addition, the past twenty years have witnessed several high-impact infectious disease outbreaks (H1N1, SARS, Ebola, Zika virus) with enormous global health burden and economic loss. In addition, regional outbreaks with viruses like Influenza A H7N9 and H5N1, Lassa fever virus, and MERS coronavirus have caused drastic local losses.[4] Improvement in infectious disease management measures is much needed to prepare for the growing threat of infectious disease, considering the absence of much-needed vaccines against viruses like HSV and low vaccination rates for viruses like Influenza virus and Ebola.[5] The key to an effective disease control measure is reducing viral loads in routes of transmission or at portals of viral entry and exit.

Ample evidence suggested that intraoral viral load is correlated with risk of disease transmission, with oral transmission 3-5 orders higher than nasal transmission.[6–9] Furthermore, the continuous infection of COVID-19, irrespective of receiving booster vaccines and the recent polio outbreaks in Israel and New York, highlights the need to address their routes of transmission.[10,11] Coronavirus, Influenza, HPV, HSV1, EBV, and KSHV viruses are transmitted orally, and their life cycle in the oral epithelium is well characterized.[12–15] New methods targeting intraoral viral load reduction are especially needed for epidemics where vaccines and therapeutics are not available. Effective transmission prevention strategy becomes very important in the scenario where almost 1 in 3 men are infected with at least one type of genital HPV and 1 in 5 men are infected with one or more types of high-risk HPV.[16] Moreover, the frequent shedding of viruses from infected individuals enhances the severity and complexity of the problem.[17] For example, HSV-1 causes frequent shedding after the first genital HSV-1 episode to up to one year.

Challenges remain for infection control regimens targeting viral load reduction in the oral cavity, with the major limitation being that most biologics can only be administered with injectable formulations. Therefore, Daniell lab has developed a new approach utilizing chewing gums through the encapsulation of protein drugs in plant cells,

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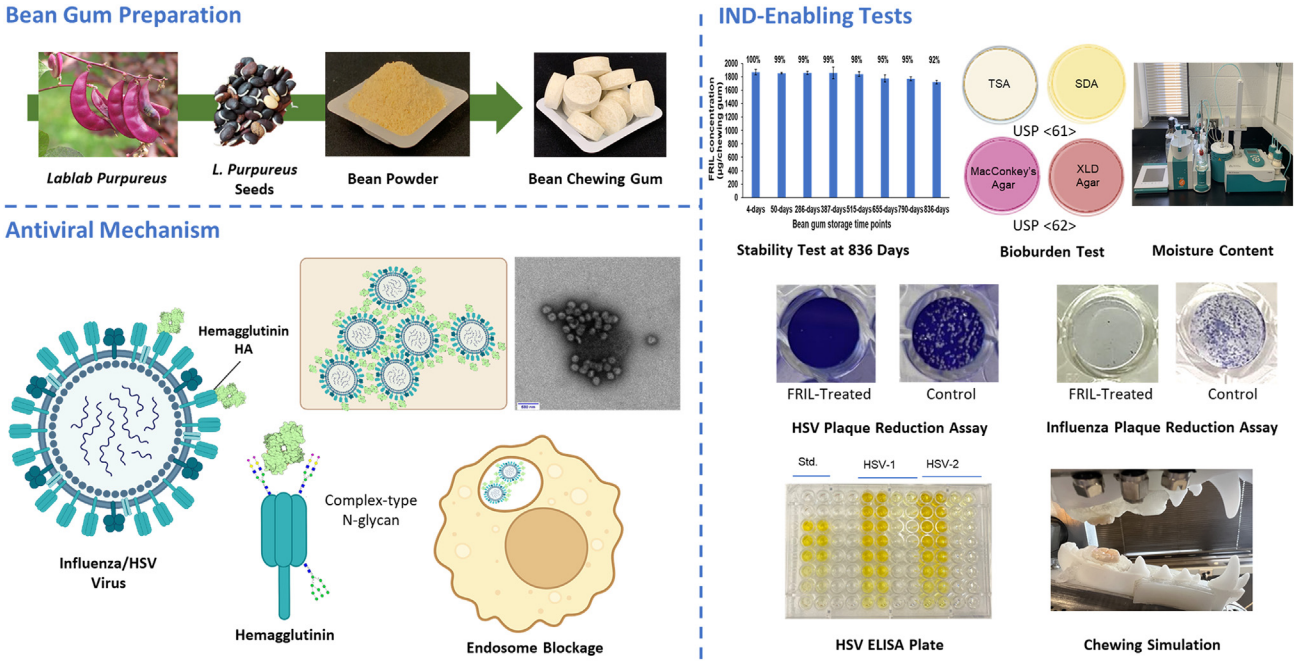

Figure 1. Graphic Abstract: Engineering and evaluation of anti-viral bean gum

allowing for their oral topical delivery. Plant cell bioencapsulation eliminates cold storage, transportation, purification challenges and significantly reduces the cost of manufacturing biologics.[18–24] The chewing gum formulation allows for effective and consistent release of the drug content at sites of viral infection. CTB-ACE2 chewing gum was able to markedly debulk SARS-CoV-2 (>95%) in COVID-19 patient saliva or swab samples.[25,26] In addition, such efficacy was consistent with various variants, including the most major beta, delta, and omicron variants. These results supported an Investigational New Drug approval from the U.S. Food and Drug Administration for a Phase I/II clinical trial (IND 154897, NCT05433181, IRB 851459), which is aimed to evaluate the viral load reduction upon chewing the gum amongst SARS-CoV-2 patients. This approach is especially suitable for orally transmitted viruses, as it could reduce viral load in saliva or the throat area, which is the main site of viral transmission.

SARS-CoV-2, Influenza virus, HPV and HSV-1 are amongst the most prevalent orally transmitted viruses. Seasonable Influenza epidemics occur annually and incur substantial disease burden worldwide.[14,27] It is estimated that more 32 million cases, 5–7 million hospitalization, and 300,000 infection-related respiratory deaths occur worldwide each year with over $11.2 billion loss annually in the United States alone.[5,27] Continuous efforts invested in vaccine development unfortunately had limited returns, as from 2004 to 2020, vaccine effectiveness among Influenza outpatients in the United States ranged from 10% to 60%.[28] Vaccination efficacy is reduced by variation in antigenic match to circulating virus strains, low vaccination coverage and disparities, and waning immunity among the elderly.[29] On the other hand, HSV-1 affects over two-thirds of the world population, with over 500,000 oral herpes cases each year in the United States alone.[30] HSV could induce encephalitis, accounting for 95% of encephalitis cases, and is the leading cause of infectious blindness in Western countries.[31] Yet, no vaccines are currently approved for HSV-1 nor HSV-2. In addition, methods beyond vaccination are needed to control the transmission of these highly prevalent viruses.

Compared to other anti-viral proteins such as monocot lectin and horcolin, Flt3 Receptor Interacting Lectin (FRIL), is significant in its anti-viral properties against a wide range of pathogens.[32] The carbohydrate-binding domain on each of four monomers of FRIL has been documented to have preferential binding affinity to complex-type N-glycans and oligosaccharides.[33] In addition, the specificity of FRIL differs from that of Man/Glc binding lectins in that the mutation in M3M6M trisaccharide allows FRIL subunits to cross-link and form higher-order structures.[32,34] Complex-type N-glycosylation is prevalent on enveloped viruses and preserved among variants as they commonly serve as the mediator for viral entry, such as the spike protein of coronavirus and the hemagglutinin of Influenza virus.[35–38] Therefore, the carbohydrate-binding domains on FRIL could bind to the complex-type N-glycans on the viral envelope and thus entrap virus in large aggregates of crosslinked FRIL and bound virion particles (Figure 1). FRIL has been documented to neutralize Influenza and coronavirus as observed through plaque reduction and microbubble assays.[24] Furthermore, HSV-1 is proposed to enter epithelial cells through four viral glycoproteins named gB, gD, gH, and gL.[36,37] Several complex-type N-linked glycans present on gB are necessary for optimal cell-cell fusion and entry.[37,38]

Utilizing the delivery platform behind the success of the CTB-ACE2 gum, in this study we developed clinical-grade anti-viral chewing gum using lablab bean powder and characterized drug product stability, bioburden, moisture content, and antiviral efficacy to neutralize Influenza (H1N1, H3N2) and HSV (HSV-1, HSV-2) viruses using plaque reduction and ELISA assay (Figure 1). Together, the bean powder chewing gum is prepared to comply with the FDA specifications for drug products and can be used to assess its potency to debulk aforementioned orally transmitted viruses. As discussed before, there is neither a therapeutic nor preventative vaccine for HSV-1 or HSV-2. Even for viruses with vaccines available, recent pandemics and epidemics have demonstrated the inefficacy of preventative measures facing mutating variants. With its unique wide-spectrum efficacy, which has been shown across viruses and variants, the bean powder gum has the potential to become a standard preventative measure against various emerging infectious diseases.

RESULTS

In this project, clinical-grade bean powder chewing gum was prepared using the same formulation and ingredients in the previously approved IND by the FDA for the ACE2 chewing gum to debulk coronavirus in saliva of COVID-19 patients (IND 154897, NCT05433181, IRB 851459), replacing the lettuce ACE2 plant powder with lablab bean powder. Both the drug substance (lablab bean powder) and drug product were evaluated for dosage, stability, moisture content, potency, and bioburden. The potency of FRIL protein and drug product (bean gum) was evaluated using two different methods (Figure 1). ELISA assay was used to evaluate aggregation of HSV-1 and HSV-2 by the bean gum to quantify virus particles remaining in the supernatant after incubation with the gum extract. Plaque reduction assay was used to evaluate the infectivity of viruses after incubation with bean gum extract or purified FRIL protein. Plaque reduction assay was evaluated in four viruses (HSV-1, HSV-2, H1N1, and H3N2). In potency assays, bean gums stored between 7 and 794 days at ambient temperature were evaluated for stability of functional efficacy.

Stability of FRIL in bean powder up to 683 days

FRIL concentration was first determined in two different stocks of lablab bean powder stored at −20°C freezer for 279 and 445 days. Optimized ELISA protocol in the lab using primary antibody developed against FRIL in rabbit and respective secondary antibody was used for estimation of FRIL in bean powder extracts of both the stocks. The quantified FRIL in stock-1 (stored 279 days) and stock-2 (stored 445 days) were 25.07 (0.07) and 24.76 (0.09) μg/mg of bean powder, respectively (Figure 2A). Less than 5% variability in FRIL concentration was observed among two different stocks stored at different time periods. The stability of FRIL in the bean powder of both stocks was further evaluated after additional storage of 238 days. The quantified FRIL in stock-1 (total storage time 517 days) and stock-2 (total storage time 683 days) were 24.49 (0.13) and 23.99 (0.24) μg/mg of bean powder (Figure 2A). During additional storage of more than 6 months (238 days) only 2.28 and 3.09% reduction was observed in stock-1 and stock-2, respectively. This confirms the prolonged stability of FRIL in the bean powder. Overall, less than 5% variability was observed in two different stocks of bean powder stored, after almost two years (683 days). These observations confirm the stability and uniformity of FRIL in different batches of bean powder.

Stability of FRIL in chewing gum up to 790 days at ambient temperature

Bean gum tablets were manufactured by Per Os Biosciences (Hunt Valley, MD). Each gum weighs 2000 mg and contains 79 mg of lablab bean powder, gum base (24.46%), sugar alcohols - maltitol (15.98%), Xylitol (1.98%), sorbitol (20.93%) and other compounds, including silicon dioxide (0.40%), isomalt (10.00%), stevia 99% (0.45%), natural flavoring agents, maltodextrin, dextrose, gum Arabic, and essential oils.

We first optimized the procedure of extracting FRIL from the chewing gum to determine FRIL dosage and stability. FRIL extraction from the chewing gum was evaluated in two different buffers, PEB and PBS, with or without Protease Inhibitor Cocktail (PIC) or other protease inhibitors in the buffer having pH similar to saliva. FRIL quantified in each bean gum tablet extracted in PEB (with PIC) and PBS (without PIC) were 1856.63 (89.81) and 1851.90 (24.85) μg per bean gum tablet, respectively, that reflects efficient extraction of FRIL from the gum tablet even in the absence of protease inhibitors and stability of FRIL in the buffer having pH similar to saliva (Figure S1). The stability of extracted FRIL in both buffers was evaluated after one cycle of freeze-thawing. Quantified FRIL concentration was 1747.10 (16.37) and 1727.37 (12.43) μg per bean gum tablet in PEB and PBS, respectively, confirming the stability of FRIL in the freeze-thawed samples (Figure S1). Two different ratios of buffer are optimized for efficient extraction of concentrated FRIL from the chewing gum. We obtained FRIL concentration up to 651.55 (15.26) ng/μL of PEB buffer (Figure S2).

FRIL concentration in the bean gum stored at ambient temperature for different time periods was determined to evaluate stability. The FRIL concentration in Protein Extraction Buffer (PEB) was 1871.12 (40.17) μg per bean gum tablet on day 4. FRIL content in gum at 4 days was considered 100% and percentage change was evaluated after prolonged storage at ambient temperature. Estimated FRIL concentrations at 4-, 50-, 286-, 387-, 515-, 655-, 790- and 836-day storage were 1871.12 (40.17), 1853.86 (7.84), 1859.51 (22.94), 1856.63 (89.91), 1838.06 (35.11), 1776.91 (53.72), 1769.00 (27.11) and 1720.56 (24.40), respectively. Less than 5% variability in FRIL concentration of chewing gum stored up to 790 days at ambient temperature confirmed stability of FRIL (Figure 2B).

Functional stability of the bean gum

As we observed that FRIL was stable in the bean gum tablets up to ∼2 years with <5% variability, the functional potency of bean gum stored at room temperature for the extended period was evaluated using H1N1 and H3N2 plaque reduction assays (1000 copies of virus per ml). The previously optimized plaque reduction assay was used for the newly manufactured (7, 37 days) and older gum tablets stored at ambient temperature (379, 753, 794, 823 days). For the Influenza

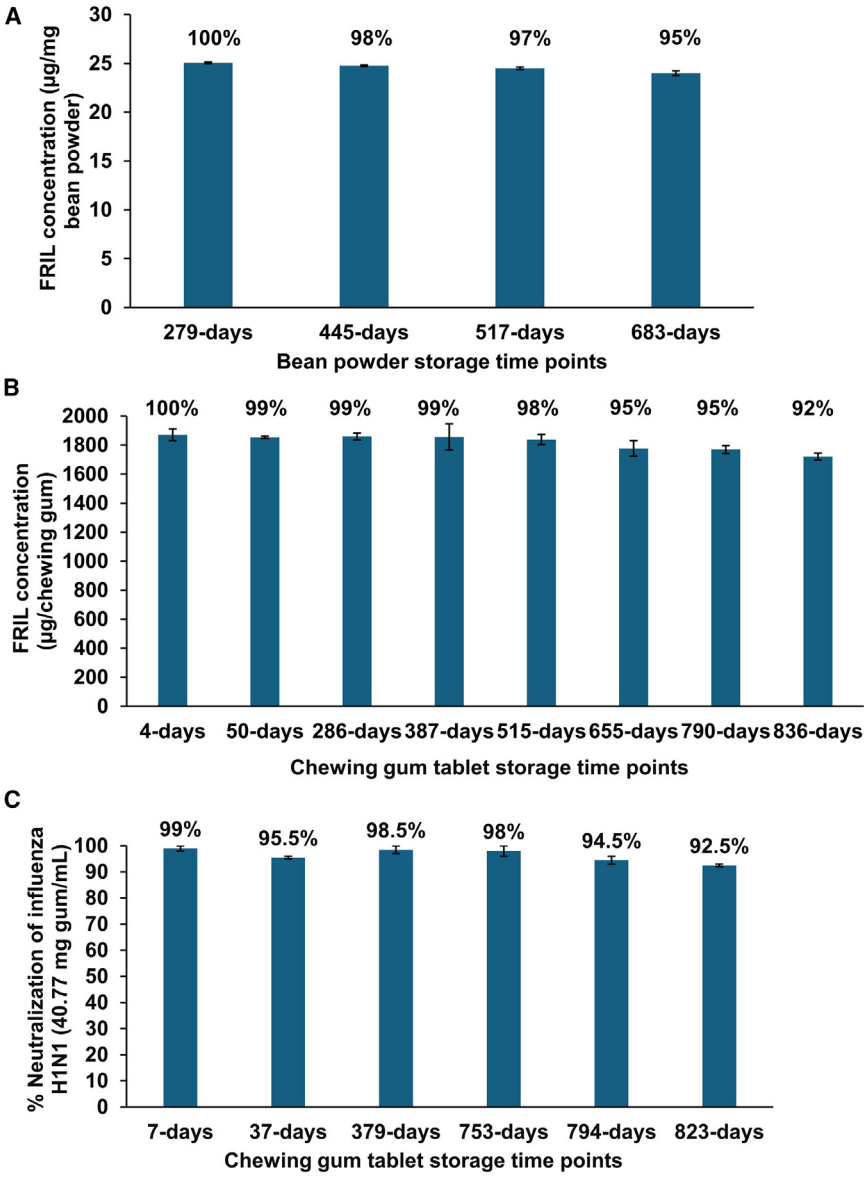

Figure 2. Evaluation of FRIL stability in bean powder (–20°C) and potency of chewing gum stored at ambient temperature for different durations

(A) FRIL stability in the lablab bean powder analyzed at 279, 445, 517 and 683 days; (B) Stability of FRIL in Chewing gum analyzed at 4, 50, 286, 387, 515, 655, 790 and 836 days. Proteins were extracted in PEB buffer (100 mM NaCl, 10 mM EDTA (pH-8.0), 200 mM Tris-Cl (pH-8.0), 400 mM Sucrose, 2 mM PMSF and 0.5× Protease Inhibitor Cocktail) and FRIL was quantified by ELISA. Data is represented as mean (standard deviation), n = 3. FRIL content at earliest time point considered 100% to compare FRIL content at later time points; (C) Influenza Virus H1N1 plaque reduction assays were performed in PBS extracts of bean gum stored at 7, 37, 379, 753, 794 and 823 days. The percentage neutralization of the H1N1 was observed at 40.77 mg/mL FRIL-gum. Data is represented as mean (standard deviation), n = 4.

H1N1 virus, >95% plaque reduction was observed at 40.77 mg/mL of gum from 7- to 753-day-old gum (Figure 2C). Please see Figure 5 below for more details on functional evaluation of both H1N1 and H2N3 virus strains. Taken together, the bean chewing gum stored at ambient temperature is functionally active for up to 2 years.

Evaluation of moisture content and bioburden

Moisture content is one of the crucial parameters in protecting drug products from contamination and enhance stability upon prolonged storage. Moisture affects the quality, shelf life, stability and safety of the drug product as high moisture content facilitates microbial growth.[25,39] To evaluate pharmacopeial compliance, bean powder and gum tablets were assessed for microbial limits and moisture content at early (<2 months) and long-term storage (~2 years) as per specified parameters for orally delivered drugs (Table.1). The drug substance lablab bean powder and bean gum passed the bioburden tests with no aerobic bacteria as well as yeasts/molds on trypticase soy agar plates and Sabouraud's agar plates, respectively after incubation of 5 days at 30°C and (USP<61>). Also, samples were tested negative for growth of *Salmonella* and *E. coli* spp. as per USP <62>. Bean gum tablets stored at room temperature for >790 days showed minimal change in moisture content (0.01%). The absence of viable microorganisms and the moisture content in lablab bean gum powder meet the requirements for clinical-grade drug products, as stated in a previously approved IND for another chewing gum product.[25,26]

FRIL release from chewing gum tablet

To determine the feasibility of topical drug release from the plant-engineered chewing gum, chewing gum tablets containing the green fluorescent protein ("GFP gum") and FRIL ("bean gum") were studied. Briefly, each chewing gum tablet contains plant materials with GFP (448 µg) or FRIL (1856 µg). After simulated chewing, release of target protein (GFP or FRIL) was quantified. Target protein release from the chewing gums were analyzed at room temperature using a physiologically comparable model: Artificial Resynthesis Technology (**ART-5**; Figure 3A).

The ART-5 is a mastication simulator that mimics human chewing motions, adapts to food texture changes, and provides immediate, reproducible computerized feedback.[40] FRIL release studies in the chewing simulator used PEB buffer that includes protease inhibitors and not human saliva. One criticism could be that proteases present

Table 1. Microbial limits and moisture content analysis of the drug substance (bean powder) and drug product (bean gum)

Test	Specification	Drug substance		Drug product	
Storage Age	–	45 Days	691 Days	7 Days	790 Days
Storage Conditions	Storage Temperature	Lot – 021722 (Stored at −20°C)	Lot – 021722 (Stored at −20°C)	Lot – 061024 (20°C–25°C)[a]	Lot – 041422 (20°C–25°C)[a]
(%) Moisture Content (USP <921>)	<10%	5.8	5.9	1.29	1.28
Microbial Limits (Bioburden) (USP <1111>, <61>, <62>)	Total aerobic microbial count < 10^3 CFU/g	0	0	0	0
	Total yeast < 10^3 CFU/g	0	0	0	0
	Total molds < 10^4 CFU/g	0	0	0	0
	Absence of *Salmonella*	Absent	Absent	Absent	Absent
	Absence of *Escherichia coli*	Absent	Absent	Absent	Absent

[a]USP Controlled Room Temperature (20°C–25°C), Excursions permitted as defined by USP (15°C and 30°C). Protect from Light.

in saliva could degrade FRIL after release. However, quantitation of FRIL was the same in PEB buffer containing protease inhibitors or PBS without protease inhibitors (Figure S1). In addition, plaque reduction assays to evaluate potency were performed using bean gum extracts in PBS buffer without protease inhibitors. Therefore, we do not anticipate any changes in release kinetics when bean gum is evaluated for potency in the clinic.

GFP gum

To optimize release kinetic assays, GFP chewing gums containing 448 μg GFP were studied (data not shown). After 45- and 60- minutes (mins) of simulated swallowing and chewing, >70% and >90% GFP was cumulatively released from the GFP chewing gum, respectively ($n = 3$). Sustained release of GFP was observed up to 60 min of chewing and continual simulated swallowing.

Bean gum

Topical protein drug release from chewing gums containing 79 mg *lablab* bean powder was analyzed ($n = 3$). During simulated chewing of the bean gum tablet in the ART-5 mastication simulator, 308.6 (10.7) μg FRIL was released in first 5 min of chewing (16.6% FRIL release rate). During the following 5 min of chewing, an additional 329.6 (15.0) μg FRIL was released (34.4% cumulative FRIL release), and after another 5 min of chewing, 353.2 (18.8) μg FRIL was released (53.4% cumulative FRIL release). The pattern of FRIL release reveals linearity during the first 15 min of chewing. Interestingly, the next 15 min of chewing released an additional 482.7 (9.8) μg FRIL (79.4% cumulative FRIL release). The following consecutive 15 min of chewing released 184.3 (7.5) (89.3% cumulative FRIL release) and 105.3 (4.9) μg FRIL (95.0% cumulative FRIL release), respectively. Therefore, the FRIL released during the last two cycles of 15-min chewing (from 30 to 60 min chewing) was reduced when compared to the first 30 min of chewing. Overall, the cumulative release of FRIL after chewing the gum for 60 min was 1763.5 (47.3) μg (95.0% release, Figure 3B). Additionally, the proportion of target protein released from both the GFP and bean gums at 45- and 60-min are comparable, indicating reproducibility of release patterns.

HSV-1 and HSV-2 aggregation by bean gum using ELISA

Potency of the bean gum tablet for HSV-1 and HSV-2 aggregation was evaluated by incubating the virus with bean gum extract and detection of virus in the supernatant after centrifugation. The rationale behind this experimental design is the ability of FRIL to aggregate viruses.[25,33] The bean gum tablets stored at ambient temperature for 352 and 375 days were evaluated for potency against HSV-1 and HSV-2, respectively. The HSV-1 viruses at 3.75×10^7 copies/mL titer were 44%, 69%, and 75% aggregated at bean gum concentrations of 33.5, 100.5, and 167.5 mg/mL respectively. While the HSV-2 viruses at 3.1×10^6 copies/mL titer were 47%, 58%, 77%, 80%, and 94% aggregated at bean gum concentrations of 16.7, 33.5, 100.5, 167.5, and 335.0 mg/mL respectively. Slightly higher percent aggregation for HSV-2 compared to HSV-1 was observed, when compared at similar bean gum concentrations (Figure 4). Considering an average 1.1 mL of saliva before swallowing in humans,[40,41] the chewing of one 2000 mg gum tablet would be ∼6 and ∼12 times more efficacious to achieve 94% and 75% aggregation of HSV-2 and HSV-1, respectively in the oral cavity. This result reflects the clinical relevance of the utilization of bean gum to prevent infection and transmission of HSV-1 and HSV-2 viruses.

HSV and influenza virus neutralization by the bean gum using plaque reduction assay

As FRIL has binding affinity to complex-type N-glycans, the presence of several complex-type N-glycans on HSV glycoproteins lays the scientific foundation of potential antiviral efficacy of FRIL against HSV, H1N1 and H3N2 (Figure 1). Certain components of bean gum interfere with the plaque reduction assay based on the viral strain and cell line used. Therefore, the placebo bean gum was evaluated first in plaque reduction assays. The placebo gum is identical to bean gum in appearance, taste, smell and composition except that it doesn't contain the bean powder. Dialysis of gum extract to remove soluble components didn't change the outcome of H3N2 plaque reduction assays using MDCK cells (Figures S3A and S3B). However, the presence of gum components reduced the number of plaques (10–20%) in HSV-1 plaque reduction assay using Vero cells at highest

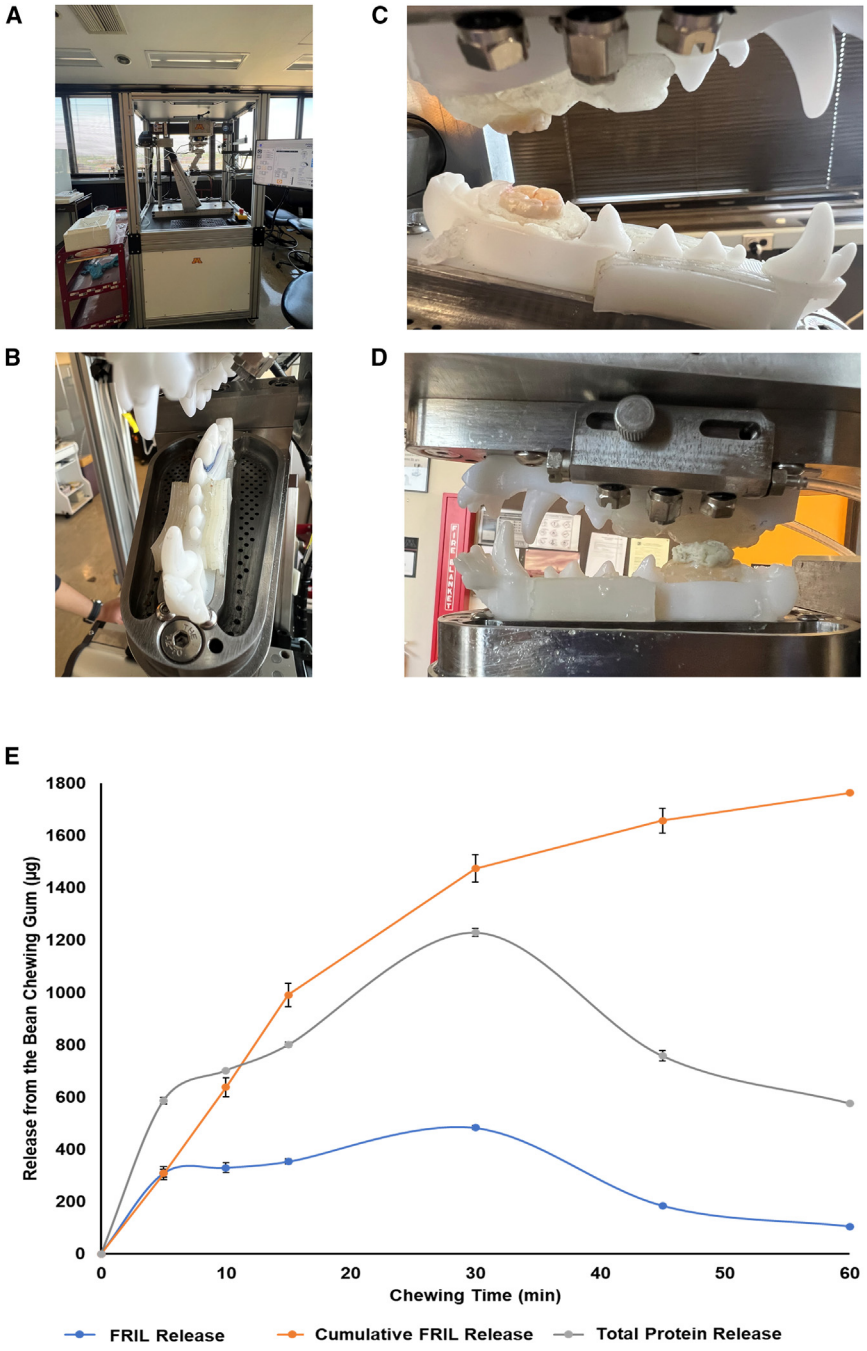

Figure 3. ART-5 mastication simulator and quantitation of total protein and FRIL released from bean gum using ART-5 mastication simulator

Overview of ART-5 mastication simulator (A) Full view; (B) top view, (C) Modified third human molars; (D) Chewing of the bean gum tablet. (E) Protein release was quantified at 0-, 5-, 10-, 15-, 30-, 45-, and 60-min of simulated chewing in PEB buffer (100 mM NaCl, 10 mM EDTA (pH-8.0), 200 mM Tris-Cl (pH-8.0), 400 mM Sucrose, 2 mM PMSF and 0.5× Protease Inhibitor Cocktail).Total proteins quantified by the Bradford assay and FRIL was quantified by ELISA. Chewing data were analyzed by a one-way ANOVA test and is represented as mean (standard deviation), $n = 3$, change in release between 0 and 60 min is significant = ****p-value <0.00001.

concentrations evaluated (Figure S3C). Therefore, all H1N1 and H3N2 plaque reduction assays were performed in gum extracts without dialysis (Figure 5). HSV-1 and HSV-2 plaque reduction assays using Vero cells were performed using dialyzed gum extracts to exclude interference of gum components (Figure 6). Therefore, any impact of components on plaque reduction assays were addressed by their removal through dialysis. All plaque reduction assays contained ∼1000 copies/mL of tested virus. For H1N1, >95% plaque reduction was observed at 40.77 mg/mL of bean gum from 7- to 753-day-old gum (Figures 5A–5D). H3N2 was assessed at two time points: ∼1 year (410-day) and ∼2 years (823-day) old gum. Over 95% neutralization of H3N2 was observed at 40.77 mg/mL of 2-year-old gum and close to 95% neutralization was observed at 40.77 mg/mL of 1-year-old gum (Figures 5F and 5G).

As the volume of saliva before swallowing in humans is an average of 1.1 mL,[41,42] we estimate that 2000 mg bean chewing gum tablet (∼50× of 40.77 mg/mL needed to reach a clinically significant reduction in viral infectivity) is sufficient to completely neutralize the two Influenza virus strains in the oral cavity. Similarly, for HSV-1 and HSV-2, we estimate the 2000 mg gum tablet in 1.1 mL saliva is ∼12× and ∼27× of concentration needed to reach a clinically significant reduction of viral load, which gives FRIL potential to be a highly efficacious anti-viral solution for oral cavity.

Antiviral potency comparison of bean gum with purified FRIL

In order to distinguish antiviral effect of gum components from FRIL present in the bean gum, we evaluated their impact independently. Purified FRIL protein showed similar level of inhibition potency with >10 µg/mL (1µg purified protein used in 100 µL treatment volume) neutralizing >90% of Influenza virus H1N1 and 95% of Influenza virus H3N2 (Figures 5H and 5I). The bean gum required 40.77 mg/mL containing 36.07–38.14 µg/mL of FRIL to achieve similar neutralization of Influenza viruses (Figures 5A–5F). Additionally, bean gum neutralized >95% of HSV-1 virus at 148 µg/mL FRIL in 159.84 mg/mL bean gum (Figures 6A and

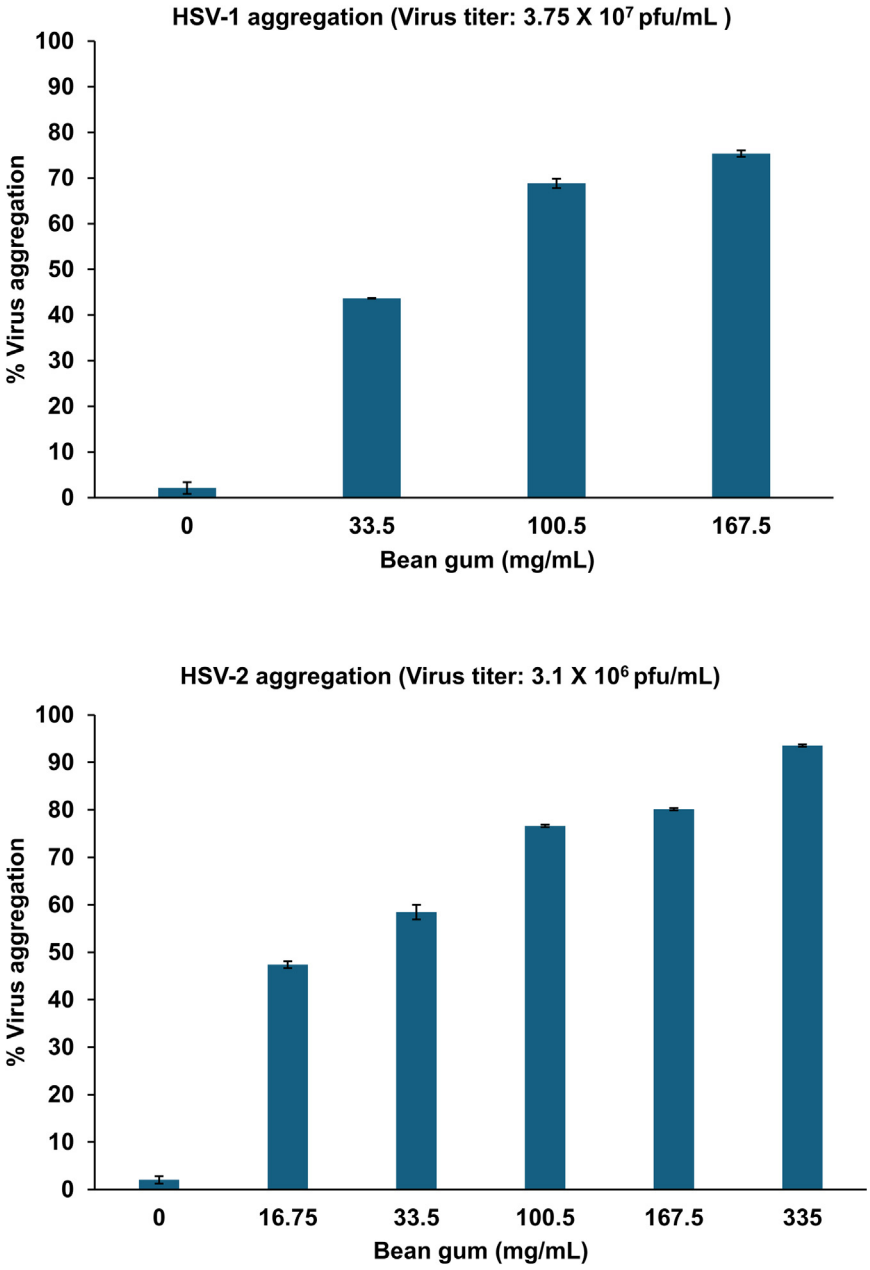

Figure 4. HSV-1 and HSV-2 aggregation at different concentrations of the bean gum extract

Virus strains were incubated with different concentrations of bean gum extract (mg/mL) for 1 h, and after centrifugation viruses in the supernatant were quantified by ELISA. Proteins were extracted in PEB buffer (100 mM NaCl, 10 mM EDTA (pH-8.0), 200 mM Tris-Cl (pH-8.0), 400 mM Sucrose, 2 mM PMSF and 0.5× Protease Inhibitor Cocktail). Virus titer used for HSV-1 and HSV-2 were 3.75 × 10^7 pfu/mL and 3.1 × 10^6 pfu/mL, respectively. Data is represented as mean (standard deviation), $n = 3$.

6B) compared to 68.52 μg/mL FRIL contained in 74.00 mg/mL bean gum against HSV-2 virus (Figures 6C and 6D). Over 95% neutralization of HSV-1 and HSV-2 viruses were observed when virus are co-incubated with purified FRIL protein at 80 μg/mL for HSV-1 (8μg purified protein used in 100 μL treatment volume) and 20 μg/mL for HSV-2 (Figures 6E and 6F). Requirement of different doses of FRIL to neutralize HSV-1 and HSV-2 is likely due to different glycosylation patterns in the glycoproteins that are key to mediating cellular entry. Several glycoproteins, such as gC and gG, are involved in the viral entry process and are absent in HSV-1.[38,43]

This comparison between bean gum and purified FRIL shows that the antiviral efficacy we observed is mainly from FRIL protein and additional anti-viral effect from other gum components is minimal, as higher concentration of FRIL is needed against both HSV and Influenza viruses. The difference between the amount of purified FRIL protein needed to reach >95% neutralization can be attributed to incomplete release of FRIL protein from the bean gum in plaque neutralization assay (incubation without stimulating release). However, 2000 mg bean chewing gum containing 1800–1900 μg FRIL should be sufficient to neutralize virus in the oral cavity. Compared to purified FRIL that requires expensive purification and cold storage, bean gum is a better choice for anti-viral treatments as it is functionally stable for up to ∼2 years when stored at ambient temperature.

Lablab bean powder safety evaluation

The presence of vicine and convicine (v-c) in lablab bean powder and chewing gum was investigated. We used faba bean (*Vicia faba* L.) as a legume species with high v-c levels in comparison to lablab products (Figure S5). A faba bean seed sample (accession ILB 938\2) with a high concentration of v-c was used in HPLC analysis in comparison to lablab bean samples. The total concentration of v-c in faba bean seed was 1.05% of seed dry matter (10,470 mg/kg). No v-c was detected in either lablab bean powder or chewing gum, ensuring the safety of the chewing gums.

DISCUSSION

There is no vaccine for HSV, but it is the most commonly transmitted, impacting 27% of American adults.[44] Unlike other viruses where symptoms are readily evident, HSV is shed and transmitted asymptomatically; HSV-1 accounts for >50% of new infections among college students and heterosexual women.[44,45] HSV-1 is universally prevalent in Africa, with an increase in seroprevalence for several

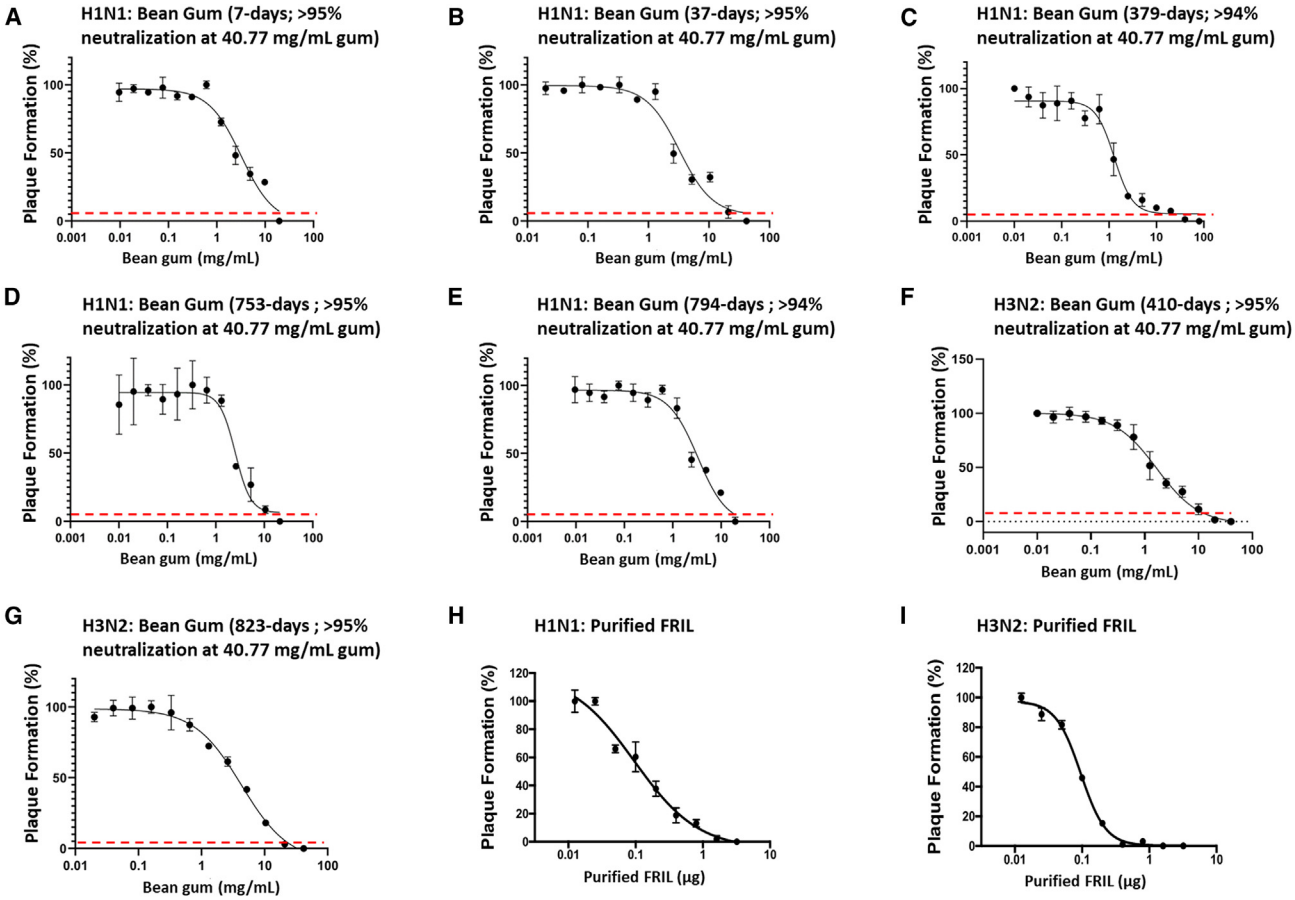

Figure 5. Functional evaluation of bean gum

Potency evaluation of Influenza virus strains H1N1 and H3N2 using bean gum tablets stored at ambient temperature for different time points: Percent neutralization of H1N1 at (A) 7-days; (B) 37-days; (C) 379-days; (D) 753-days; (E) 794-days. Percent neutralization of H3N2 at (F) 410-days; (G) 823-days. The red line shows 95% neutralization. Purified FRIL evaluated against H1N1 (H) and H3N2 (I) is also shown for comparison. All gum extracts were prepared in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH-7.4). All assays were performed using viral titer of ~1000 copies/mL. Data is represented as mean (standard deviation), $n = 4$ for H1N1 and $n = 6$ for H3N2.

decades, mostly through oral-to-oral transmission.[45] HSV-2 seroprevalence is high among HIV patients (65% in the US, 85% in Africa among heterosexuals)[46]; unfortunately, anti-retroviral therapy doesn't reduce HSV-2 acquisition[47] and HSV has already developed resistance to widely used antiviral drugs.[48] As reported by Moin et al.,[47] HSV prevalence is high throughout the globe: 55–67% of the population (28% had oral lesions) in Spain, Germany, and France; In Asia, much higher HSV-1 seroprevalence was observed in India, China, and Japan ranging from 88% to 96%; In Africa (Egypt and Morocco) the number is 98–99%, and in South America 63 to 83% for HSV-1 and HSV-2; In Australia, HSV-1 infection increased 16% in 2017 from 45% in 2004.

Herpes labialis is the most common orofacial form of infection, known as cold sores, and HSV is primarily transmitted through lesional fluids, saliva, and even though brief kissing from a father to a child.[47–49] Although HSV-2 was believed to be not primarily transmitted orally, due to increase in oral sex prevalence, HSV-2 shedding through oral cavity is now more common among the general population. Although HSV-2 is the leading cause of recurrent genital herpes, genital HSV-1 is now known to affect millions of individuals in the US and is currently the leading cause of newly diagnosed genital herpes infection in the US.[50] Recently analyzed saliva samples of 220 individuals revealed that HSV-2 is more commonly shed in the oral cavity than HSV-1.[51] However, HSV-2 oral shedding has been documented for several decades among HIV-positive men, again due to oral sex.[52] HSV-2 shedding through oral mucosal lesions has also been documented for several decades.[53] These statistics underscore the need to reduce transmission of HSV.

Although lack of vaccine is pointed out as a limitation for HSV, most recent vaccines confer protection from the severity of the disease but do not prevent infection or transmission. This controversy was widely debated in the public press during the recent COVID-19 pandemic.

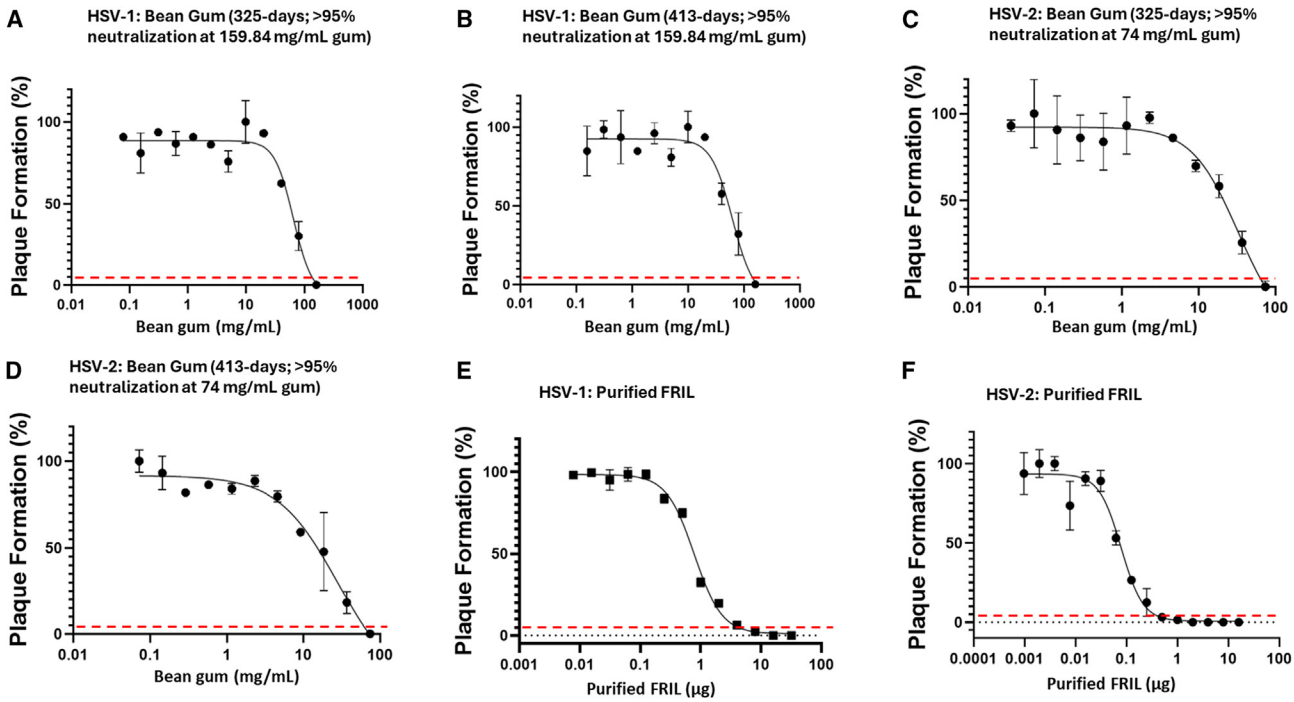

Figure 6. Potency evaluation of bean gum tablet using HSV-1 and HSV-2

HSV-1: Over 95% neutralization observed at 159.84 mg/mL bean gum containing 148.00 µg/mL FRIL; Experiments performed with (A) 325-day (B) 413-day old gums. HSV-2: Over 95% neutralization observed at 74.00 mg/mL bean gum containing 68.52 µg/mL FRIL; Experiments performed with (C) 325-day (D) 413-day old gums. Purified FRIL evaluated against HSV-1 (E) and HSV-2 (F) is also shown for comparison. The red line shows 95% neutralization. All gum extracts were prepared in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH-7.4), and dialyzed in PBS buffer overnight to reduce the concentration of sugar alcohols and other inferring compositions of bean gum. All assays were performed using viral titer of ~1000 copies/mL. Data is represented as mean (standard deviation), $n = 4$ for bean gum against HSV-1 and HSV-2, $n = 2$ for purified FRIL protein against HSV-1 and HSV-2).

Fully vaccinated individuals have breakthrough COVID-19 infections, and the peak viral loads are similar to unvaccinated individuals and efficiently transmit viruses in household settings.[6] The scientific explanation is that recent vaccinations confer systemic immunity that predominantly produced IgG and not secretory IgA (mucosal immunity) that neutralizes viruses at their points of entry. Global use of oral polio vaccine that offers both mucosal and systemic immunity has demonstrated the importance of vaccines to prevent transmission, in addition to conferring protection.[54] All current Influenza vaccines develop systemic immunity and, therefore, do not prevent transmission. Airborne volume of saliva droplets in healthy subjects is 3–5 orders of magnitude higher than breath droplets and speaking four words transmits more virus than 1 h of maskless breathing.[55] Therefore, in this study we evaluate the ability of the antiviral protein to reduce viral load delivered using chewing gum, to facilitate slow release of antiviral protein for better efficacy in the oral cavity.

To facilitate the evaluation of bean gum in human clinical trials, we prepared clinical-grade drug product and characterized them to meet regulatory requirements. FRIL is stable in the ground bean powder when stored at −20°C up to 683 days. Storage of powder is not necessary because lablab been seeds could be ground from seeds whenever required. However, to meet regulatory requirements, drug substance (bean powder) was stored to evaluate dosage and stability. During this process, we optimized different buffer conditions and protective agents, including protease inhibitors because plant cells release abundant proteases in crude extracts. Fortunately, none of these stability agents were required because FRIL was stable in PBS buffer without any additives. The stability of FRIL in plant powder is not unique because we have previously observed stability of human, bacterial, or fungal proteins in freeze-dried plant cells for several years when stored at ambient temperature.[19–26,56,57]

FRIL is stable when gum is stored at ambient temperature for up to 790 days. This observation is not surprising because we have observed such long-term stability of proteins in several other chewing gums products (up to 3 years in GFP chewing gum).[21] Bean gums used for Plaque reduction assay were stored for 7–823 days at ambient temperature. Chewing gums contain the same ingredients and percentages as the gum in previously approved IND (gum base, sugar alcohols, components). The drug substance lablab bean powder and bean gum passed the bioburden test with no aerobic bacteria as well as yeasts/molds, following USP <61>, <USP 62> guidelines for evaluation, like previously approved IND requirements. The moisture

content for both bean powder and bean gum tablet is 5.9% and 1.28% respectively, which is within the acceptable range of <10%. The absence of viable microorganisms and moisture content in lablab bean powder and the bean gum powder meets requirements for clinical-grade drug products, based on previously approved IND for another chewing gum product.[25]

Mimicking that of human swallowing, the ART-5 continually removes and introduces liquids and proteins while chewing, suggesting that results are physiologically relevant. Since the bean gum showed steady and significant release FRIL, the bean gum is suitable for evaluation of self-infection as swallowing saliva is critical to neutralizing viruses in the throat surface. Moreover, the majority of FRIL is released from the chewing gum tablets after only 15 min of simulated chewing (53.4% cumulative FRIL release). Complete inhibition of ∼1,000 copies/mL H1N1, H3N2, HSV-1, and HSV-2 viruses are observed at 38.14 μg/mL, 38.14 μg/mL, 148 μg/mL, and 68.5 μg/mL of FRIL protein, through the plaque reduction assays. As discussed earlier, ∼475 μg/mL FRIL was released within the first 5 min of chewing. While the potency of bean gum could vary depending on virus variants and the viral titer in the patient's oral cavity differs from individual to individual, we believe significant neutralization could be achieved within 5 min of chewing. Additionally, continual FRIL and total protein released at each time point, despite continuous removal of solution, highlights the feasibility of sustained topical FRIL delivery while chewing (Figure 3E). Such observation lays the foundation clinical evaluation.

Mechanistically, FRIL has been shown to exhibit a homogeneous tetramer structure with four carbohydrate-binding domains (CBD). The four CBDs are identical and facilitate binding of FRIL to complex-type N-glycans.[32,33] In addition, FRIL facilitates crosslinking to form higher-order structures through protein-protein interactions like van der Waals interactions.[33] Therefore, FRIL interaction with complex-type N-glycans, through virus binding and self-binding to form large aggregates of entrapped viruses prevents virus from entering the host cell and escaping from the endosome upon entry. The potency of bean gum was evaluated using two different methods. ELISA was used to evaluate aggregation of HSV-1 and HSV-2 by FRIL to quantify virus particles remaining in the supernatant after incubation with the bean gum extract. The total volume of saliva droplets is several orders of magnitude higher than breath droplets.[9] Therefore, trapping virus particles in chewing gum is a key step in prevention of oral virus transmission. Bean gum extracts trapped HSV-1 up to 75% at virus titer 3.75×10^7 pfu/mL and HSV-2 up to 94% at virus titer 3.1×10^6 pfu/mL, in a dose-dependent manner. ELISA measures only virus aggregation but plaque reduction assay measures both viral aggregation and blockage of H1N1, H3N2, HSV-1, HSV-2 at the endosomes by FRIL in host cells.[25,32] Therefore, lower inhibition (∼75%) is observed in ELISA whereas >95% inhibition is observed in plaque reduction assays. In addition, percentage inhibition between these assays is not comparable because plaque reduction assays were done with 1,000 viruses per ml (amount reported in the oral cavity), whereas ELISA assays are done with 1–10 million viruses per ml in order to increase absorbance signal.

Therefore, plaque reduction assay was used for evaluation of aggregation and blockage of virus not trapped by FRIL at the endosomes inside cells after entry.[32] This assay shows not only aggregation to trap viruses but also the prevention of infection. Therefore, plaque reduction assay was evaluated in four viruses (HSV1, HSV-2, H1N1, H3N2). Virus neutralization (>95%) was observed at ∼40 mg/mL of bean gum with H1N1 and H3N2, when ∼1000 copies/mL of viruses were used in plaque reduction assays. Influenza virus titer in infected patients beyond 2 days after symptom onset, as measured by viral RNA concentrations, is reported to be 3.62 (2.13) $\log_{10}$ copies/mL.[58] Because each gum tablet weighs 2000 mg and only ∼40 mg/mL is required for >95% neutralization, bean gum tablet has 50-fold higher than efficacy dose and therefore it is suitable for evaluation in the clinic.

For HSV-1, 160 mg of bean gum resulted in >95% inhibition of plaque formation without negative impact on cell proliferation, in dialyzed gum extracts to remove interference from components. For HSV-2, >95% inhibition of plaque formation was observed with 74.00 mg/mL. Differential neutralization efficacy between HSV-1 and HSV-2 is most likely due to different glycosylation patterns of surface glycoproteins. For example, Glycoprotein G is glycosylated in HSV-2 but not in HSV-1.[59] Higher glycosylation of HSV-2 may correlate with lower dose of FRIL required for neutralization, but further mechanistic studies are needed. For HSV-1 virus, viral DNA titer greater than 2.3 $\log_{10}$ copies/mL is generally considered positive. For HSV-2 virus, quantities greater than 4 $\log_{10}$ DNA copies/mL have been modeled as leading indicator to interpersonal transmission.[17] Considering errors that could occur from RT-PCR measurement, we believe our experiment design provides a close simulation to viral titer of infected patients' saliva. Each bean gum tablet (2000 mg) has 27-fold for HSV-2 and 12.5-fold higher for HSV-1 neutralization efficacy and is therefore suitable for clinical evaluation.

HSV-1 KOS strain used in our study is a clinical isolate collected from Kendall O. Smith, a well-known virologist at Baylor College of Medicine, Houston TX.[60] KOS 63 and variants are of Asian origin and KOS 79 strain is common in North America and Europe.[61] HSV – strain 333 also originated from Texas.[62] Both these strains are extensively used in various preclinical and clinical investigations. While monovalent glycoprotein D conferred protection against HSV challenge,[63] clinical evaluation utilizes trivalent mRNA vaccine containing three different glycoproteins.[64] In our virus trap investigations, ELISA assays used antibodies against both B2 and D2 glycoproteins, confirming that FRIL interacts with both these glycoproteins, which are common to most HSV clinical strains and play a critical role in viral entry into host cells.[65] Dose dependent reduction by FRIL shows efficient binding to highly conserved glycoproteins, thereby predicting efficacy against several clinical strains despite their variability among other surface proteins.

Lablab bean powder is Generally Regarded as Safe (GRAS notice No GRN 000879, 2020) by the FDA based on toxicology reports submitted.[66] Genetic diversity among *Lablab purpureus*, faba, or fava beans is minimal. Evaluation of simple sequence repeat markers shows high similarity among different beans, showing their common genetic ancestry.[67] The primary difference is the presence of glucosides vicine/convicine in Faba beans that could be harmful to glucose-6-phosphate dehydrogenase (G6PD) -deficient patients causing favism.[68,69] However, lablab bean has no detectable levels of vicine/convicine (Figure S4), and therefore is safe for consumption.

Lablab is a versatile crop. Lablab dry seeds are prized for their nutritional value: rich in protein, fiber, vitamins, and minerals.[70] In African and Asian countries, Lablab consumption is promoted to address malnutrition.[71–74] It originated in tropical Asia and Africa and was cultivated in India as early as 1500 BC.[75] Its consumption as food spans centuries and continents, with its origins traced back to Africa, particularly around Ethiopia, where it was first cultivated for its edible seeds and pods. From there, it gradually spread to other regions, including tropical Asia. Today, lablab is cultivated in various tropical and subtropical regions worldwide, including parts of South America and Australia.[76]

The safety and efficacy of bean powder have been studied in several animal models. Intranasal administration of 2.9 mg or 29 mg/kg/day purified FRIL was well-tolerated and therapeutic when administered to H1N1-infected mice.[32] Several safety studies have been conducted using bean powder in mice and rats. IP administration of 30 mg/kg (60× above the gum dose) was well-tolerated in a mouse tumor model.[77] As reported in the GRAS determination,[66] Schmandke et al. conducted 4-week feeding studies of several different processed bean powder formulations in male and female Shoe:Wist/Il rats (30 rats per group, dosage 20, 40, and 80 g/kg).[78] The low dose of 20 g/kg was considered the no-observed-effect level (NOEL). Compared to the gum, lablab bean dose of ∼0.0007 g/kg in humans, this rat NOEL is more than the target clinical dose by several orders of magnitude. The dose of lablab bean powder in the bean gum is 79 mg, when compared to 500 g ingested in human clinical toxicology evaluation.[69] This is 6,330-fold less than what would be consumed as food. Chewing gum has evolved over 9000 years, transitioning from chewing resin and sap directly from trees to medicated gums containing aspirin, nicotine, or vitamins.[79]

Interestingly, FRIL preserves hematopoietic progenitors[80] and neural progenitors,[81] and has been used to prolong *in vitro* maintenance of quiescent human cord blood CD341CD382/low/SCID repopulating stem cells.[82] Cells cultured with FRIL engraft differentiated into erythroid myeloid and lymphoid lineages in the bone marrow of both primary and secondary transplanted recipients. Unlike other plant lectins, FRIL does not induce cell proliferation or IL-6 secretion.[82]

Seasonal Influenza viruses expelled from the oral cavity contribute to viral transmission in both low- and high-humidity environments.[83] Several antiviral products have been evaluated in the clinic to reduce viral load and symptoms. Iota-carrageenan is a polymer derived from seaweed and traps viruses.[84] Although initial studies reported positive results,[85] reanalysis of randomized trial data of Carrageenan nasal spray showed a recovery rate of 54%, much lower than >200% observed for Zinc lozenges in five different clinical trials.[86] Echinaceae/Salvia lozenges derived from plants showed a reduction in viral load but required very high doses - 16,800 mg per day plant extracts, resulting in higher adverse events.[87,88] Nasal spray of Iota-carrageenan in COVID-19 patients showed significant efficiency in preventing COVID-19 in healthcare workers managing patients with this disease.[89] Careful evaluation of Zinc or vitamin C in COVID-19 patients had negligible impact on infection.[90] Lozenges containing amylmetacresol, 2,4-dichlorobenzyl alcohol or hexylresorcinol had no measurable antiviral activities.[84] Furthermore, broad-spectrum antiviral chemicals result in viruses developing resistance, which is especially a major challenge to control HSV.[91] Several mouthwashes with virucidal effects were reported.[92] Several sprays containing antiviral products were marketed as OTC products during COVID-19 pandemic, but the FDA published warning notices for not providing proof for clinical efficacy for those products. Therefore, there is a great need to develop efficacious oral topical antiviral drugs. FRIL protein is efficacious at very low concentrations by trapping viruses like broad-spectrum chemicals but also enters human cells and blocks viruses at the endosomes. Chewing gum as a delivery vehicle is preferable to lozenges because of slow and prolonged release of drug substances[79] but direct comparative studies of delivery methods using the same antiviral product are needed. Clinical evaluation of different delivery methods (lozenges, spray, tablets, drops) didn't result in measurable outcomes, although authors recommended orally delivered formulations.[88]

A phase 3 study to assess the efficacy of the antiviral drug baloxavir to evaluate household transmission of Influenza is in progress (NCT03969212).[93] A very large study in 208,225 families with participants in different age groups showed that oral antiviral delivery is better than inhalers to control household viral transmission.[93] Evaluation of different neuraminidase inhibitors showed that household transmission was highest with oseltamivir.[94] Although Peramivir or Zanamivir neuraminidase inhibitors are effective, they are delivered intranasally or intravenously.[95,96] However, our study focuses on oral delivery. Oseltamivir is the most widely used anti-Influenza drug globally and is orally delivered.[96,97] A detailed analysis of 2247 citations on non-pharmaceutical interventions (hand washing, disinfection, oral hygiene, physical barriers, etc.) did not reveal any conclusive benefits.[97] Topical delivery of biologics to control virus infection and transmission is an emerging new area of research.[79,98] Chewing gums containing antiviral proteins are efficient in significantly (>90%) reducing viral load of several SARS-CoV-2 strains (Beta, Delta, Omicron) in saliva samples of COVID-19 patients.[25,26] Similar *ex vivo* clinical studies were not feasible for influenza or Herpes patients because ongoing vaccine studies do not evaluate mucosal samples. Injectable vaccines confer protection through IgG and not secretory IgA that could block viruses at their points of entry and

prevent transmission.[54,99,100] Chewing gums containing enzymes disrupt the dental plaque and kill bacteria and yeast that cause dental caries.[22] Preparation and CMC characterization of bean gum and potency to neutralize HSV1, HSV-2, H1N1 and H3N2 viruses in this study augur well for advancing this product to the clinic to minimize infection or oral transmission.

MATERIALS AND METHODS

Estimation of FRIL in lablab bean powder

We received lablab (hyacinth) bean (*Lablab purpureus* (L.) Sweet) seed powder from Dr. Che Ma (Genomics Research Center, Academia Sinica, Taiwan) and estimated FRIL concentration as described earlier[32] with some modifications. Lablab bean seed ground powder (100 mg) suspended in 1 mL of protein extraction buffer (PEB, 100 mM NaCl, 10 mM EDTA (pH-8.0), 200 mM Tris-Cl (pH-8.0), 400 mM Sucrose, 2 mM PMSF and 0.5× Protease Inhibitor Cocktail), was mixed properly through vortexing, and incubated for 1 h at 4°C on homogenizer (Vortexer). The solution is then sonicated 6 cycles (10 s on and 15 s off) at 80% amplitude and spun at 750 × g for 5 min at 4°C. Supernatant was collected and quantity of soluble FRIL (Flt3 Receptor Interacting Lectin) was estimated by ELISA. In ELISA, 100 μL of bean powder extract supernatant or purified FRIL protein as standards was coated in the wells of microtiter plate (Corning Incorporated, Costar Assay Plate 3590, NY, USA) and incubated overnight at 4°C. Next day, after washing with PBST (PBS +0.05% Tween 20) four times, the blocking was done with PBST having 3% non-fat dry milk for 1 h at 37°C. After three washes with PBST, the plate was incubated with 100 μL of anti-FRIL (primary antibody developed in rabbit) antibody (1:2000), at 37°C for 1.5 h. Plate was then washed three times with PBST, and incubated with 100 μL of anti-Rabit IgG raised in goat conjugated with HRP (1:5000), incubated at 37°C for 1.5 h. Finally, plate was washed three times with PBST and 100 μL of HRP substrate (TMB/E Solution, Mllipore Carp.) was added in each well. The plate was incubated for 2 to 10 min at RT depending on developed color intensity. Reaction was stopped by adding 50 μL of 2N H_2SO_4 and absorbance measured at 450 and 700 nm using microplate reader (BioTek, Synergy H1, VT USA). Additional absorbance at 700 nm measured to normalize any possible absorbance due to the turbidity of the extracts. FRIL protein was estimated by comparing standards. Experiments were performed in triplicate and data presented as mean (standard deviation).

Chewing gum preparation from the lablab bean powder

Chewing gum tablets containing ground lablab bean seed powder were prepared by Per Os Biosciences (Hunt Valley, MD), via compression process, allowing for homogeneous availability of protein drugs without raising the temperature. The bean gum tablets were prepared with the following excipients – gum base (24.46%), sorbitol (20.93%), maltitol (15.98%), Xylitol (1.98%), magnesium stearate (3.00%), silicon dioxide (0.40%), isomalt (10.00%), stevia 99% (0.45%), natural flavoring agents (dextrose, maltodextrin, essential oils, gum arabic) to make the gum tablets flavorful, and conducive to compression. The physical architecture and flavor of the prepared bean gum tablets were similar to other non-therapeutic chewing gums available in the market made by conventional methods that use high temperature and extrusion. The received gum tablets from Per Os Biosciences were stored in the mylar bags to avoid moisture absorption and light exposure. Few tablets were kept in the Uline black containers for routine examination viz; bioburden, moisture content, drug dose quantitation and release.

Estimation of FRIL in the prepared chewing gum

Estimation of FRIL in the prepared chewing gum of lablab bean powder was done after extracting total soluble proteins in two different buffers (i.e., Phosphate Buffer Saline (PBS) and PEB. A bean gum tablet of 2000 mg was crushed and ground into the mortar and pestle. Afterward, 334 mg grinded material was suspended in 1 mL of PBS or PEB, mixed properly, and incubated for 1h at 4°C on homogenizer (Vortexer). Samples were then sonicated 9 cycles (10 s on and 15 s off) at 80% amplitude and spun at 750 × g for 5 min at 4°C. The supernatant was then collected, and soluble FRIL quantified by ELISA. Following the same protein extraction and ELISA protocol, stability of FRIL in chewing gum extracts stored in freezer and single time thawed were determined. Experiments were performed in triplicates and data represented as mean (SD), $n = 3$.

Protein release from GFP and bean gum tablets

The feasibility of protein-impregnated chewing gum tablets for topical application was evaluated by the protein released after simulated chewing and swallowing through the ART-5 mastication simulator. To replicate swallowing while chewing within the oral cavity, the ART-5 was programmed to continually introduce and remove liquids at an average flow rate (PEB introduced) of around 100 μL (μL) per minute of chewing. The angle of bite ranged between 0° and 13°, resulting in an extension of 11.6 mm. Similar to human chewing speeds,[101] an average of 1.00 Hz (Hz) was coded into the chewing software. To facilitate physiologically comparable results, the ART-5 was modified to include sound upper and lower 3rd human molars. After taking acrylic impressions, the extracted molars were ground to 2 mm (mm) of enamel on occlusal surfaces of the maxilla and mandible and mounted into the ART-5 using resin (Figure 3A).

To improve the ability of the 2-g chewing gum tablets to stick to the sound human molars in the ART-5 machine during early chewing, tablets were pre-soaked in 500 μL PEB buffer for 30 min. Over this 30-min interval, >90% PEB was absorbed by the chewing gums, and total protein released from the chewing gum tablets from this incubation step was minimal (<5%). The pre-soaked chewing gum tablets were then placed onto the mandible of the ART-5 machine, with simulated swallowing and chewing motions commencing afterward. The buffer swallowed after chewing was collected and stored on ice at 0-, 5-, 10-, 15-, 30-, 45-, and 60-min. Protein released from the chewing gums were then analyzed at the aforementioned timepoints. Release from the GFP gum was quantified by using known concentrations of the recombinant GFP protein (Cell Biolabs STA-201). For the GFP chewing gums, the samples retrieved after simulated swallowing were then loaded into a 96-well black plate (Corning Incorporated, Costar Assay Plate 3916, ME USA). The fluorescence intensity was

read on a multi-mode plate reader (BioTek, VT) at 485 nm (excitation) and 538 nm (emission). For the bean gums, the released FRIL was quantified using ELISA as described previously using the anti-FRIL antibody as the primary antibody and its respective secondary antibody.

HSV-1 and HSV-2 aggregation with bean gum extracts

In HSV aggregation assay, bean gum protein extract of different concentrations in protein extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-cl (pH-8), 400 mM Sucrose, 2 mM PMSF and 0.5× PIC) incubated with Virus (HSV-1 and HSV-2 titer were 3.75×10^7 pfu/mL and 3.03×10^6 pfu/mL, respectively) in presence of 100 mM $MnCl_24H_2O$ for 1h at 37°C with intermittent mixing. FRIL supernatant without virus and virus without FRIL supernatant used separately as control. After incubation, spun at 14000 rpm for 10 min. Supernatant collected and evaluated for the presence of virus by the ELISA. In ELISA assay, 50 μL of supernatant coated into wells of ELISA plate and incubated for 3 h at RT. After three washes with PBST, blocking was done with 200 μL of 5% non-fat dry milk in PBST overnight at 4°C. Wells washed three times and incubated with primary antibodies (anti-glycoprotein B and anti-glycoprotein D of HSV-1 or HSV-2 raised in rabbit, mixed, each 5 μg/mL) and incubate for 2 h at RT. After three washes secondary antibody (goat anti-rabbit IgG conjugated with HRP) added and plate incubated for 2 h at RT. After three washes with PBST, 100 μL of substrate (TMB/E Solution, Mllipore Carp.) added and incubate for 2–10 min at RT for color development. The reaction was stopped by adding 50 μL of 2 N H_2SO_4, and absorbance measured at 450. After subtracting the absorbance of respective controls, percent of virus remained into the FRIL treated sample was calculated. Accordingly, inhibition was calculated. Aggregation experiment performed in triplicates and data represented as mean (SD).

Moisture content analysis by Karl Fisher coulometry

Moisture content assessment was done as per USP <921> Method I (coulometry). Titrando 860 kF Thermoprep and Titrando 851 were used and the titration parameters viz; temperature and pump flow rate were set to 150°C and 50 mL/min on the Titrando 860 kF Thermoprep respectively. The instrument was monitored for constant drift of 10.0 (5.0) μg/min before performing any estimations. This is the important step for precise measurement of moisture content. To measure the residual water content of moisture present in the empty glass vial, it was sealed with aluminum cap and used for titration. Three such titrations were performed to measure blank to get averaged value for final calculation. For measurement of standard, 50.0 (2.0) mg of HYDRANAL-water standard powder was weighed in glass vial (Cat. Code 34693, Honeywell Specialty Chemicals Seelze GmbH, KF Oven 150°C–160°C) and capped with aluminum stopper immediately. Two such vials were prepared and assessed for moisture content. The exact weight of water standard was recorded in the parameters section of the KF machine. The instrument was calibrated using blank in triplicates and water standard in duplicates. The standard should be in the range 4.96–5.04% for hydranal at the specified parameters. For measuring sample, bean gum tablet was homogenized in mortor pestle and 50 mg of the gum tablet powder was weighed in glass vial stoppered with aluminum seal and bottle was inserted on KF coulometer for moisture content. Similarly, 50 mg lablab bean powder was weighed in glass bottle, capped with aluminum seals and analyzed for moisture content.

Bioburden assessment

The lablab bean powder and bean gum powder were analyzed for bioburden test was done as per United States Pharmacopeia guidelines USP <61>, <62> to assess aerobic bacterial and yeast/mold counts and presence of pathogenic bacteria viz; *Salmonella* and *E.coli* respectively. For aerobic bacterial and yeast/mold counts sterile Trypticase Soy Agar (TSA- Becton Dickinson) and Sabouraud's Dextrose agar plates (SDA - Oxoid) were used respectively. Bean powder was directly used for preparation of suspension for microbial enumeration. bean gum tablet was transferred aseptically to pre-sterilized mortar and pestle and homogenized to obtain uniform powder for preparation of suspension for microbial enumeration. Ten mg of each sample was weighed aseptically in sterile Eppendorf tubes and sterile phosphate buffered saline was added to prepare serial dilutions for counting colony forming units/mL Suspension was thoroughly vortexed to get uniform suspension. Each dilution was spread inoculated (0.1 mL) onto TSA and SDA plates and kept face up in biosafety cabinet. TSA and SDA plates were incubated at 37°C and 25°C respectively for 5 days. For detecting pathogenic bacteria *E. coli* and *Salmonella* spp., samples were inoculated onto MacConkey's Agar and Xylose lysine decarboxylate agar respectively as per USP<62> and incubated at 37°C for 48h. Uninoculated plates were used as negative control to ensure sterility of media.

Cell line culture

Madin-Darby Canine Kidney (MDCK) cells and African green monkey kidney epithelial Vero cells and Vero E6 cells were used in plaque reduction assay. Cell lines were maintained in an incubator with 5% CO_2 at 37°C. MDCK cells were grown in Minimum Essential Media Alpha (MEM α) medium, with the addition of 5% heat-treated fetal bovine serum (FBS, Sigma), 2 mM L-glutamine, 100 units/mL penicillin, 1.25 μg/mL of amphotericin B (Fungizone), 50 μg/mL gentamicin, 10 mM HEPES and 100 μg/mL streptomycin. Vero and Vero E6 cells were grown in same medium with Dulbecco's modified Eagle's medium in replacement of MEM α medium.

Plaque reduction assay

Bean gum powder was evaluated for their abilities to prevent infection of Influenza virus strains H1N1 (A/California/7/2009-X181) and H3N2 (A/Singapore/INFMH-16/0019/2016), Herpes Simplex virus 1 (KOS strain) and Herpes Simplex virus 2 (333 strain) using a quantitative viral plaque reduction assay.

Influenza virus

Prior to the assay, MDCK cells were plated in 48-well plates and incubated for 24 h to reach 80–100% confluency. 330 mg Finely ground bean gum powder was mixed with 1mL PBS, sonicated on ice to release soluble FRIL protein and centrifuged. The supernatant was

collected as sonicated bean gum extract. The sonicated bean gum extract was diluted with PBS to reach various concentrations of FRIL, and the Influenza strains was added to reach 1 pfu virus/μL of Influenza. The samples were incubated at 4°C on a rocker for 1 h, then added onto MDCK cells in 48-well plates for infection. The plate was incubated with 5% CO2 at 37°C for 1 h, then the virus mixtures were aspirated and washed to eliminate un-absorbed virus. The cells were overlaid with serum-free MEM medium supplemented with 0.3% Avicel, 0.3% Methylcellulose, 1× TPCK-treated trypsin, and 0.1% BSA. The cells were incubated with 5% CO_2 at 37°C for 34 h, fixed and immune-stained with anti-Influenza nucleoprotein antibody. Viral plaques were microscopically counted and analyzed to generate dose-response curves.

Herpes simplex virus

Sonicated bean gum extract was dialyzed overnight to remove sugar alcohols and other inferring compositions of bean gum. HSV virus was co-incubated with increasing concentrations of sonicated bean gum extract to reach 1 pfu virus/μL of HSV-1 or HSV-2. The samples were incubated at 4°C on a rocker for 1 h, then added onto Vero cells in 48-well plates. Vero cells were then infected by adsorbing 100 μL of the HSV FRIL mixtures at 34°C for 45 min, then the virus mixtures were aspirated and washed to eliminate un-absorbed virus. The cells were overlaid with serum-free DMEM medium supplemented with 0.3% Methylcellulose. The cells were incubated with 5% CO_2 at 37°C for 50 h for HSV-1 and 40 h for HSV-2, fixed and stained in 4% formaldehyde and 0.2% crystal violet. Viral plaques were microscopically counted and used to generate dose-response curves.

Quantitation of vicine and convicine in lablab bean powder and bean gum

Determination of vicine and convicine in faba beans by HPLC-DAD. Vicine and convicine were determined based on the method described by Gutierrez et al.[102] Briefly, the samples were extracted with water in a hot water bath for 3.5 h. Concentrated HCl was then added to the sample extracts. Samples were analyzed by an Agilent 1100 series high-performance liquid chromatography with a diode array detector (HPLC-DAD). For the separation of compounds, a C-18 -column was used with a gradient of phosphate buffer and methanol.[103] Vicine standard was purchased from Sigma -Aldrich (St. Louis, MO, USA).

Statistical analyses

Data of bean gum total dose quantification, release, ELISA, and plaque reduction assay was presented by means (standard deviation). Statistical significance was calculated by One-Wau Electrochemical impedimetric measures with an average of three duplicates. GraphPad Prism 9.2 was used to plot and analyze graphs. The plaque count was quantified manually using a dissecting microscope.

DATA AND CODE AVAILABILITY

The raw data required to reproduce the above findings will be available from the corresponding authors upon request.

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AUTHOR CONTRIBUTIONS

HD conceived this project, designed experiments, interpreted data, participated in discussions and wrote/edited several versions of this manuscript. YG performed assays on evaluation of purified FRIL, bean gum, and placebo gum potency against H1N1, H3N2, HSV-1, and HSV-2, participated in discussions, created the graphic abstract, and wrote the introduction section of the paper. UK performed H1N1, H3N2 plaque reduction assays using bean gum stored at different intervals of time and wrote results. RS participated in determination of FRIL stability in bean powder and bean gum, and quantification of FRIL release from the chewing in simulated chewing. RS performed HSV-1 and HSV-2 aggregation using ELISA assays, analyzed and wrote relevant sections. RS provided chewing gum extract for plaque reduction assays. RJK performed release kinetic studies for GFP and bean gums, analyzed and interpreted data, and wrote corresponding sections. GW conducted bioburden and moisture content assessments for bean powder and bean gum tablets as per USP guidelines. HK and J-MP performed vicine-convince analysis and interpreted data and wrote corresponding sections. GHC designed viral trap assays, provided HSV virus strains and developed antibodies required for these assays, reviewed several versions of this manuscript and helped in addressing questions from reviewers. YG, RS, RJK, GW, and HK wrote results section of corresponding sections.

DECLARATION OF INTERESTS

HD is a patentee in plant-based oral and topical drug delivery and was previously funded by Novo Nordisk, Bayer, Shire and Takeda. Several industry discussions for advancing clinical trials in oral or topical delivery are in progress. Complete list of patents is available in the Google Scholar or ScholarGPS links below:

http://scholar.google (in PBS buffer).com/citations?user = 7sow4jwAAAAJ&hl = en

https://scholargps.com/scholars/82094026790000/henry-daniell.

SUPPLEMENTAL INFORMATION

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