Title: Inactivation mechanism and efficacy of grape seed extract for Human Norovirus surrogate

Running title: Grape seed extract inactivates Tulane viruses

Chamteut Oh[1,*], Ratul Chowdhury[2,*], Laxmicharan Samineni[3], Joanna L. Shisler[4], Manish Kumar[6], and Thanh H. Nguyen[1,5]

[1]Department of Civil and Environmental Engineering, University of Illinois at Urbana-Champaign, USA
[2]Department of Chemical Engineering, The Pennsylvania State University, PA, USA
[3]Department of Chemical Engineering, The University of Texas at Austin, Austin, USA
[4]Department of Microbiology, University of Illinois at Urbana-Champaign, USA
[5]Institute of Genomic Biology, University of Illinois at Urbana-Champaign, USA
[6]Department of Civil, Architectural and Environmental Engineering, The University of Texas at Austin, Austin, USA

Equal contribution
Corresponding author(s): Thanh H. Nguyen (thn@illinois.edu)

34 **Abstract**

35 Proper disinfection of harvested food and water is critical to minimize infectious disease. Grape

36 seed extract (GSE), a commonly used health supplement, is a mixture of plant-derived polyphenols.

37 Polyphenols possess anti-microbial and -fungal properties, but antiviral effects are not well-known.

38 Here we show that GSE outperformed chemical disinfectants (e.g., free chlorine and peracetic

39 acids) in inactivating Tulane virus, a human norovirus surrogate. GSE induced virus aggregation,

40 a process that correlated with a decrease in virus titers. This aggregation and disinfection were not

41 reversible. Molecular docking simulations indicate that polyphenols potentially formed hydrogen

42 bonds and strong hydrophobic interactions with specific residues in viral capsid proteins. Together,

43 these data suggest that polyphenols physically associate with viral capsid proteins to aggregate

44 viruses as a means to inhibit virus entry into the host cell. Plant-based polyphenols like GSE are

45 an attractive alternative to chemical disinfectants to remove infectious viruses from water or food.

46

47 **Keywords:** polyphenols; grape seed extract; Human norovirus surrogate Tulane virus; virus

48 aggregation; Molecular docking simulations

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Importance

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51   Human noroviruses are major food- and waterborne pathogens, causing approximately 20% of all

52   cases of acute gastroenteritis cases in developing and developed countries. Proper sanitation or

53   disinfection are critical strategies to minimize human norovirus-caused disease until a reliable

54   vaccine is created. Grape seed extract (GSE) is a mixture of plant-derived polyphenols used as a

55   health supplement. Polyphenols are known for antimicrobial, antifungal, and antibiofilm activities,

56   but antiviral effects are not well-known. In studies presented here, plant-derived polyphenols

57   outperformed chemical disinfectants (e.g., free chlorine and peracetic acids) in inactivating Tulane

58   virus, a human norovirus surrogate. Based on data from additional molecular assays and molecular

59   docking simulations, the current model is that the polyphenols in GSE bind to the Tulane virus

60   capsid, an event that triggers virion aggregation. It is thought that this aggregation prevents Tulane

61   virus from entering host cells.

62

Introduction

63

64   Human noroviruses cause approximately 20% of all cases of acute gastroenteritis in
65   developing and developed countries (1). In the United States, human noroviruses cause about 5.5
66   million cases of foodborne illnesses per year and about 2 billion dollars in economic loss (2, 3).
67   Noroviruses are transmitted primarily by the fecal-oral route, including ingestion of contaminated
68   food and water or via person-to-person contacts (4). Thus, inactivating human noroviruses present
69   in contaminated food or water is important.

70   Sodium hypochlorite (NaClO) is regarded as the cheapest and most effective disinfectant
71   to inactivate noroviruses on produce surfaces (5), stainless steel surfaces (6), or in liquid solutions
72   (7). Fresh and fresh-cut produce are generally sanitized with residual chlorine concentrations of
73   50-200 μg/mL (8). Unfortunately, NaClO is toxic, and produces carcinogenic disinfection by-
74   products (9–11). Thus, both consumers and industry are seeking methods to naturally or minimally
75   processed foods with minimal/zero chemical additives (12, 13). Some organic compounds such as
76   peracetic acid or vinegar have been studied as alternative disinfectants. Fuzawa et al. (2020) found
77   that the Tulane viruses were significantly more resistant to peracetic acid than rotaviruses because
78   the Tulane viruses were aggregated due to the low pH of peracetic acid (i.e., pH 3). These authors
79   concluded that further optimization is necessary to improve the inactivation efficacy (14). Five
80   species of bacteria (*C. albicans, S. mutans, S. aureus, E. coli,* and *B. subtilis*) were treated with
81   100% vinegar for 10 min and 0.5 to 4.0-fold reduction in CFU/mL was found depending on
82   bacteria species (15). However, vinegar's virucidal efficacy and mechanism have not been fully
83   understood.

84

85   Plants and fruits synthesize polyphenols, chemicals that protect them against damage from

86   external stresses such as infection. Grape seed extract (GSE), a by-product of grape juice and wine

87   production, is mass-produced at an affordable price (16, 17). GSE is sold as an FDA-approved

88   health supplement (18). GSE polyphenols have antimicrobial effects (19) and are considered

89   popular alternatives to chemical disinfectants. GSE possesses antiviral activity against feline

90   calicivirus (FCV-F9), murine norovirus (MNV-1), bacteriophage MS2, and hepatitis A virus (16,

91   20, 21). However, the mechanism of virus inactivation is unclear (18). This information is critical

92   because, if GSE is to be used by industries as a natural disinfectant, then researchers must

93   understand how GSE inactivates different types of viruses.

94   The objective of this study was to examine GSE-induced inactivation of Tulane virus, a

95   surrogate for human norovirus. Tulane virus is an ideal surrogate for norovirus because of its

96   structural similarities to human noroviruses (22–24). Thus, Tulane virus has been used to provide

97   more information about the potential inactivation of norovirus (7, 14, 25–28). Once GSE

98   disinfection was established, we identified the mechanism of this inactivation using both molecular

99   assays and computer modeling. We found that GSE's main disinfection action occurred due to

100   virus aggregation. In addition, we conducted molecular docking simulations to identify potential

101   interactions between GSE polyphenols and viral capsids. This study is the first observation that

102   shows plant-derived polyphenols can outperform chemical disinfectants in safely and sustainably

103   controlling waterborne and foodborne pathogens. This study also provides the mechanism for

104   GSE-induced virus inactivation. Thus, GSE may be an attractive disinfectant for finished products

105   such as drinking water and foods because of its safety, efficacy, and lower environmental impact.

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107

Results

GSE outperforms chemical disinfectants by 3-log$_{10}$ TV titer reduction

Fig. 1 shows results from the examination of disinfection properties of GSE against Tulane virus, using a range of different GSE concentrations, Tulane virus (TV) concentrations, and incubation times. For all experiments, GSE was incubated with purified TV. GSE activity was halted by the addition of FBS, and plaque-forming units (PFUs) were quantified as a measure of virus inactivation. We tested GSE concentrations ranging from 42 μg/mL to 678 μg/mL because similar GSE concentrations were shown to inactivate other enteroviruses (16, 20, 21).

Fig. 1

TV inactivation increased when GSE concentration and duration of incubation increased (**Fig. 1A**). The inactivation curves were fitted to both the pseudo-second-order model and Chick's law. The correlation coefficients (R^2 values), which reflect the goodness of fit, obtained by the pseudo-second-order model (0.99 to 1.00) were higher than those by Chick's law (0.34 to 0.54) except for the lowest GSE concentration (42 μg/mL) where no significant reduction in virus titers was detected (one-way ANOVA, $p > 0.05$). Parameters from the inactivation kinetics experiments are also listed in **Supplementary Table 1**. The fitting to the pseudo-second-order model showed that it took less than 120 seconds to reach a 95% of log$_{10}$ PFU reductions at equilibrium state (i.e., $t_{95} < 120$ seconds) for all tested conditions except for the 42 μg/mL case.

In **Fig. 1A**, better fitting achieved by the pseudo-second-order model suggested that chemisorption is the dominant reaction between the polyphenols and virus particles (29–31). This finding suggested there were optimal GSE:virion ratios for disinfection, as supported by data in

130   **Fig. 1B** and **1C**. **Fig. 1B** showed that TV inactivation increased as GSE concentrations increased,

131   with GSE efficacy reaching a plateau at 424 μg/mL. This plateau in GSE efficacy suggests a

132   maximum number of sites on the virions where GSE can bind to. Therefore, GSE concentration

133   needs to be optimized for a given virus concentration. **Fig. 1C** showed that GSE disinfection

134   efficacy significantly depended on the initial virus titer. Specifically, GSE disinfection efficiency

135   decreased when more TVs were added to a reaction, supporting the hypothesis that chemisorption

136   occurs between GSE and TV. Interestingly, while 42 mg/mL GSE showed no disinfection activity

137   for 10^6 PFU TV (in **Fig. 1A**), it indeed possess antiviral properties when lower numbers of TV are

138   present, further supporting the model that GSE polyphenols adsorb to TV surfaces. We compared

139   GSE inactivation kinetics to those of two commercially available chemical disinfectants (i.e., free

140   chlorine and peracetic acid) using a widely accepted disinfection model (i.e., Chick's law) and the

141   rate constants obtained from a previous study (7, 14) (red and green lines in **Fig. 1D**). We found

142   42 μg/mL of GSE inactivated the 10^5 PFU/mL of TV to a 3-$\log_{10}$ virus titer reduction within 16

143   seconds, a time identical to that of inactivation by free chlorine. In contrast, peracetic acid took

144   much longer (211 seconds) to achieve the same levels of inactivation.

145

146   **GSE causes viral aggregation, and this is likely the main mechanism of GSE-induced**

147   **virus inactivation**

148   **Fig. 1** data showed that GSE inactivated TV and GSE adsorbed to the virion surfaces. Thus,

149   one possibility is that GSE binds directly to TV to prevent virus-host interactions. **Fig. 2A**

150   quantifies GSE polyphenols in the absence or presence of TV as a means to examine if GSE indeed

151   binds to TV. We incubated 847 μg/mL GSE with 10^6 PFU TV. The 2 mL mixture was then

152   subjected to ultracentrifugation to separate free versus virus-bound GSE, and GSE in supernatants

7

153   of samples were quantified. As shown in **Fig. 2A**, there was a statistically significant lower GSE

154   concentration in supernatants when TV was present, implying that GSE indeed binds to TV. Next,

155   we used a light scattering analyzer (DelsaMax Pro, Beckman Coulter) to determine the particle

156   size distributions of intact TV and GSE (**Fig. 2B)**. Untreated TV showed a polydispersed

157   multimodal size distribution with a major peak at 97 nm and two relatively smaller peaks at 309

158   and 1755 nm. A single TV virion has a diameter of 40 nm (32), thus most of the TVs in solution

159   were present as dimers, and some populations of viruses were present as timers. Virus populations

160   at 1755 nm were likely multi-virus aggregates. When TV was incubated with 15 μg /ml GSE, there

161   was a shift in the size of the TV peak at 94 nm to approximately 300 nm and 1000 nm, implying

162   that this concentration of GSE causes aggregation of TV into trimers and perhaps decamers.

163   Experiments using higher concentrations of GSE resulted in greater shifts in sizes, suggesting that

164   GSE is causing TV aggregation. GSE showed a strong single peak at around 1000 nm regardless

165   of GSE concentrations. This peak is believed to represent insoluble polyphenols that are self-

166   aggregated (33). When the GSE solutions were filtered with 0.1 μm filter, the monodispersed peak

167   disappeared from the particle size distribution.

168

Fig. 2

169

170   To further test if GSE induces virus aggregation, we quantified virus aggregation and virus

171   titers in parallel and compared the numbers of virus aggregates and inactivated virus particles after

172   the GSE treatment. As shown in **Fig. 2C**, 10^6 PFU TV was incubated with the indicated

173   concentrations of GSE (85 to 847 μg/mL) for 2 minutes followed by quenching the reaction with

174   FBS. Viruses were then either quantified by plaque assay or tested using an aggregation assay.

175   Aggregation assays were designed as follows: untreated or GSE-treated TV were subjected to

176   filtration using a 0.1 μm sized pore filter. Because a single TV virion has a diameter of 40 nm (32),

177   we expect that single and dimeric TV would easily filter through the pore while virus aggregates

178   with three or more virions would be trapped in the filter. Note that our aggregation assay cannot

179   determine a specific number of virions in virus aggregates that can pass through the filter. Viruses

180   that passed through the filter were quantified by one-step RT-qPCR. The ratio of genome copies

181   for untreated versus treated viruses (Log_{10} gc_0/gc) were plotted on the y-axis in **Fig. 2C**. Similar

182   to **Fig. 1**, plaque assays measured virus titers in untreated samples (PFU_0) versus GSE-treated

183   samples (PFU), and data are presented as the ratio of virus titers before and after exposure to GSE

184   (Log_{10} PFU_0/PFU) on the y-axis in **Fig. 2C**. Virus aggregates and plaque were very similar when

185   GSE concentrations below 400 ng/ml were used. However, virus aggregates were significantly

186   lower than inactivated virus titers when 847 μg/mL GSE was present. This observed discrepancy

187   shows that the number of virus particles larger than 0.1 μm does not fully explain the number of

188   inactivated virus particles determined by the plaque assay. Nevertheless, paired t-test revealed no

189   significant difference between the plaque assay and aggregation assay results over the different

190   GSE concentrations ($P>0.05$). Thus, our current model is that GSE interacts with TV capsid,

191   causing TV aggregation. In turn, aggregation would likely prevent TV from entering host cells.

192   We also demonstrate that GSE aggregation was not reversible; removal of GSE from TV-

193   containing solutions did not make aggregated TV return back into single particles

194   (**Supplementary Text 1**).

195

196   **Molecular docking indicated polyphenols strongly bound to capsid proteins**

197   Data from **Fig. 2** indicated that GSE is physically associated with TV and induced virus

198   aggregation. This event likely prevents TV from entering the host cell to begin the virus life cycle.

199   To further investigate this possibility, we conducted molecular docking simulations, an approach

200   used to identify interactions between polyphenolic compounds and proteins (34, 35). GSE is a

201   mixture of various polyphenolic compounds (36, 37). We selected ten polyphenolic compounds

202   present in GSE for molecular docking experiments. We chose these compounds based on

203   abundance (37), and this included two phenolic acids (gallic acid and protocatechuic acid), one

204   stilbenoid (resveratrol), three monomer flavan-3-ols (catechin, epicatechin, and epicatechin

205   gallate), and four dimer flavan-3-ols (procyanidin B1, B2, B3, and B4) based on LC-MS

206   metabolite profiling analysis and UHPLC-QqQ-MS analysis (**Supplementary Table 2**). Although

207   we conducted inactivation experiments using TV, we could not perform molecular docking

208   simulations with the TV capsid because there is no entry for the TV capsid in the Protein Data

209   Bank (PDB, https://www.rcsb.org/). However, Cryo-EM analysis has shown similarity between

210   the TV and human norovirus (HuNoV) capsids (32), and the HuNoV capsid structure is present in

211   the PBD. Thus, we studied the interaction of the GSE polyphenols with the HuNoV VP1 capsid

212   proteins to understand how GSE may interact with similar TV capsid proteins.

213   We performed flexible molecular docking simulations (38, 39) between each of the ten

214   GSE polyphenolic compounds and HuNoV VP1 proteins to discern stable docking conformations

215   across the four domains of VP1 (S, S-P1 hinge, P1, and P2 domain; **Supplementary Fig. 2**) to

216   identify where each of the polyphenols tended to bind in order of domain preference. For these

217   simulations, we used the HuNoV icosahedral asymmetric unit (PDB ID: 1IHM) that comprised

218   three VP1 proteins because the complete HuNoV capsid consists of 180 identical icosahedral

219   asymmetric units. Residue-level domain definitions have been described by Campillay-Véliz et al.

220   (40). Flexible molecular docking simulations are shown in **Fig. 3**. The expected binding strength

221   of each polyphenol (in increasing order of size/molecular weight) with HuNoV capsid protein has

222   been reported where the error bars indicate variance across top 15 polyphenol binding poses within

223   the same capsid groove. The scale on the right indicates fractional capsid-binding affinity of a

224   given polyphenol with respect to the positive control – BSA. Larger polyphenols tend to show

225   increased affinity towards both the positive control and target capsid of HuNoV. Analysis of

226   binding affinities from docking experiments reveals greater electrostatic stabilization of the larger

227   conjugated electron systems within the bigger polyphenols by the polar grooves of HuNoV capsid

228   and BSA alike. Data demonstrated that each of these ten polyphenolic compounds likely bounds

229   to different residues or regions of the HuNoV capsid protein. In addition, these polyphenols

230   appeared to bind to a location at the dimeric interface of the trimeric capsid protein. The scores

231   obtained from the simulation reflect the enthalpic contribution of binding (kcal/mol) between the

232   polyphenols and the capsid (41). Fifteen independent trajectories of docking were performed using

233   the docking protocol from OptMAVEn-2.0 suite (39) and the Rosetta energy function (42) was

234   used to score the docked poses. The expected binding score was the modal value (i.e., most

235   probable score) across the fifteen recorded values per complex. Since the polyphenols tend to show

236   a conjugated electron system, larger polyphenols showed better electrostatic stabilization by the

237   polar binding groove offered by the capsid proteins. Consequently, flavan-3-ol dimers showed

238   stronger binding affinities to the capsid protein than smaller groups of polyphenols (**Fig. 3**). The

239   polyphenol-capsid interaction profiles were composed primarily of hydrogen bonds along with

240   hydrophobic interactions, accounting for the efficient capsid capture seen in experiments (**Fig. 4**).

241   For example, the smallest polyphenol protocatechuate establishes three hydrogen bonded and three

242   hydrophobic contacts while procyanidin (the largest polyphenolic compound) shows up to ten

243 hydrogen bonded contacts yet maintaining the same number of hydrophobic interactions with the

244 capsid (**Supplementary Fig. 3**). In addition to being concordant with a previous experimental

245 study showing that polyphenols' binding affinity to proteins (e.g., BSA and human salivary α-

246 amylase) increased with their molecular weights (43), we also provide key biophysical insights for

247 the same in this work (**Fig. 4**). Binding affinities for respective polyphenols with the HuNoV

248 capsid were near-equal to that of BSA, our positive control (**Fig. 3**). BSA is known as the major

249 protein in FBS (44), with reported quenching activity towards polyphenolic compounds. Thus, our

250 results indicated that the GSE polyphenols tend to promote capture of viral capsids with high

251 efficiency.

252

Fig. 3

253

Fig. 4

254

255 It is noteworthy that as the size of the polyphenol increases, it tends to bind to a less-buried,

256 more solvent-exposed pocket (owing to steric clashes) (**Fig. 5**) yet binding to the interface of two

257 chains of the trimeric capsid (located around the S-P1 hinge domain; **Supplementary Fig. 2**). Only

258 the largest Procyanidin (B1-B4) polyphenols cannot access the inter-chain interface pockets and

259 are primarily surface binders with most interactions limited to a single chain (**Fig. 5**). We thereby

260 hypothesize that larger polyphenols tend to be surface binders but stronger capsid binders, while

261 smaller ones preferably bind to inter-chain interfaces of the viral capsid. In terms of domains bound,

262 our docking simulations, in concordance with the experimental data, show that most polyphenols

263   bind to the S or S-P1 hinge domains - while trace binding is observed in the P1 domain and P2

264   domain is mostly unbound.

265

Fig. 5

266

267   We extended the molecular docking simulations to other enteric viruses such as feline

268   calicivirus (FCV-F9), murine norovirus (MNV-1), and hepatitis A virus (HAV) that were

269   experimentally studied in previous works (16, 20, 21). Although the three sets of experiments were

270   performed in different conditions, such as different virus media (water or produce) and different

271   pH, they presented a similar trend that FCV-F9 is more susceptible to the polyphenols in GSE than

272   MNV-1 and HAV. Our molecular docking simulation agreed with the experimental data with these

273   enteric viruses. The binding energy of the polyphenols to FCV-F9 capsid protein was stronger than

274   those of MNV-1 and HAV ($P<0.05$) (**Fig. 6**). Note that the binding energy alone does not give

275   sufficient information to evaluate the antiviral effect of the polyphenols. Therefore, *in vitro*

276   experiments are still required to determine virus inactivation efficacy and mechanisms.

277

Fig. 6

278

279   **Discussion**

280   Anti-bacterial, anti-biofilm, and antifungal effects of plant-derived polyphenols are well-known.

281   In this study, we systematically evaluated the antiviral efficacy and mechanism of the polyphenols

282   in GSE to TV, a surrogate virus for HuNoV. Data shown here suggest that GSE can inactivate TV

283   and perhaps other enteric viruses. In addition, data show that the polyphenols in GSE aggregate

284   the virus particles, an event that is likely responsible for TV inactivation. Indeed, high binding

285   energies between polyphenols and capsid proteins calculated by molecular docking analysis

286   supported the GSE-induced TV aggregation (Fig. 6). Our current model is that TV aggregation

287   prevents proper virus-host interactions or virus entry into host cells. At least one other study has

288   reported that aggregation of herpes simplex virus prevents virus entry into cells while allowing

289   virus attachment (45).

290   We found antiviral efficacy of GSE depends on initial virus titers. It should be noted that

291   the TV concentrations used in this study are likely much higher than those found in contaminated

292   produce or water. For example, rotavirus concentrations in river water, treated wastewater, and

293   untreated wastewater are reported to be $10^{-3.0}$,$10^{-2.2}$, and $10^{-1.3}$ FFU/mL, respectively (46, 47).

294   Given that GSE was efficacious for these artificially high TV concentrations in the experiments

295   presented here, it is reasonable to expect that GSE would be effective as an antiviral for

296   contaminated food or produce. Also, TV is more resistant to chemical disinfectants than the other

297   viruses in the *Caliciviridae* family (48). Thus, it is likely that GSE would be efficacious against

298   other caliciviruses, and this is an avenue of future research. GSE is also attractive because it has

299   been approved for consumption by humans. Thus, GSE or plant-derived polyphenols could be

300   advantageous over the chemical disinfectants for drinking water and foods.

301   The experiments conducted in this study have several limitations. Thus, further research is

302   necessary before GSE is widely used to inactivate water-borne and food-borne pathogens. First,

303   GSE consists of numerous polyphenolic compounds at different concentrations, and its

304   constituents depend on various factors such as grape species, cultivation regions, and extraction

305   methods. Therefore, GSE products may have different polyphenol compositions and antiviral

306   efficacy. We did not perform inactivation experiments with single polyphenol compounds, which

14

307   is important information to estimate the antiviral efficacy of the other GSE products. Future studies

308   could perform a set of inactivation experiments with purified polyphenol compounds and

309   determine their contributions to antiviral activity. Second, we conducted only *in vitro* experiments,

310   and *in silico* analyses, so a future direction is to identify the effective dosage of GSE (and its active

311   compounds) to begin to understand the actual mode of action in detailed mechanisms. In addition,

312   although GSE is a health supplement approved by the US Food and Drug Administration (FDA),

313   its side effects cannot be ignored. For example, GSE might enhance antiplatelet effects when

314   combined with other supplements (49). Finally, water contains other organic matter which could

315   outcompete viruses for binding to polyphenolic compounds. For example, Joshi et al. (16) showed

316   that the antiviral efficacy decreased when milk with high protein content was added to an

317   inactivation reaction. Thus, further studies need to be performed to understand how effective GSE

318   is in water with different compositions and under various physical conditions. Other water

319   properties (e.g., pH, ionic strength, and temperature) are important for adsorption reactions, and

320   additional studies need to address how these properties may affect the efficacy of GSE. If GSE or

321   individual polyphenols were to be used for foods (e.g., apples) then we must also understand how

322   the surface properties of foods (e.g., wax contents of produce surface) may affect the efficacy of

323   GSE (14).

324

325   **Materials and Methods**

326   **Commercially available grape seed extract and its plant-derived polyphenols**

327   We purchased a commercial grape seed extract (GSE) solution for this research (7832, Natures

328   plus, USA). The total polyphenol (TP) concentration in each GSE sample was quantified by the

329   colorimetric method using Folin-Ciocalteu reagent. A mixture of 100 µL GSE sample and 500 µL

330   of 10% Folin-Ciocalteu phenol reagent (Sigma-Aldrich, MO, USA) was prepared in a 10 mm path

331   length polymethyl methacrylate (PMMA) cuvette (BrandTech Scientific, CT, USA). Within 3 to

332   8 min of mixture preparation, 400 μL of 7.5% sodium carbonate solution was added to the mixture

333   and homogenized by pipetting. After incubating at room temperature for 60 minutes, UV

334   absorbance at 765 nm was measured by a spectrophotometer (UV-2450, Shimadzu, Japan).

335   Distilled water was used as a reference solution. The same procedure was conducted with gallic

336   acid solutions with different concentrations prepared by diluting gallic acid monohydrate (Sigma-

337   Aldrich, MO, USA) in distilled water. The TP concentrations of the gallic acid solutions were used

338   for a calibration curve (**Supplementary Fig. 4**) which determined TP concentration of the GSE

339   samples. GSE concentrations ranging from 84 to 1694 μg/mL were prepared by diluting the initial

340   GSE from 25-fold to 500-fold with 1X PBS and used for inactivation experiments. PBS is widely

341   used as a vehicle for *in vitro* virus experiments (7, 28, 50). Note that the Folin-Ciocalteu reagent

342   can react with any reducing substances other than polyphenols. We applied the same colorimetric

343   method to TV solutions and found that virus concentrations of 10^6 PFU/mL, the highest virus titer

344   used in this study, are equivalent to 18±1 μg/mL of GSE concentration. To exclude the impact of

345   TV on GSE concentration determination using this assay, we determined GSE concentration by

346   multiplying the initial GSE concentration, which did not contain TV, by dilution factors.

347   GSE and four standard compounds (catechin, procyanidin B2, gallic acid, and resveratrol)

348   were analyzed with the Triple 6500+ LC/MS/MS system (Sciex, Framingham, MA) in

349   Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-

350   Champaign. Software Analyst 1.7.1 was used for data acquisition and analysis. The 1260 infinity

351   II HPLC system (Agilent Technologies, Santa Clara, CA) includes a degasser, an autosampler, and

352   a binary pump. The LC separation was performed on an Agilent SB-Aq column (4.6 x 50mm, 5μm)

353 with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in

354 acetonitrile). The flow rate was 0.3 mL/min. The linear gradient was as follows: 0-2min, 95%A;

355 10-15min, 0%A; 15.1-20min, 95%A. The autosampler was set at 10°C. The injection volume was

356 10 μL. Mass spectra were acquired under negative electrospray ionization (ESI) with the ion spray

357 voltage of -4500 V. The source temperature was 400 °C. The curtain gas, ion source gas 1, and ion

358 source gas 2 were 35, 50, and 65 psi, respectively. Multiple reaction monitoring (MRM) was used

359 for quantitation: gallic acid (from m/z 169.0 to m/z 79.0), catechin (from m/z 289.0 to m/z 205.0),

360 procyanidin B2 (from m/z 577.2 to m/z 299.0); resveratrol (from m/z 277.0 to m/z 185.0).

361 We also characterized the GSE using UHPLC-QqQ-MS with four standard compounds of

362 catechin, procyanidin B2, gallic acid, and resveratrol. We chose these four compounds to represent

363 each subgroup of polyphenols (i.e., monomers flavan-3-ol, dimers flavan-3-ol, phenolic acid, and

364 stilbenes). We discovered catechin, procyanidin B2, gallic acid, and resveratrol account for 5.97,

365 2.96, 0.67, and 0.001% of total polyphenols in the GSE. Although we could not quantify every

366 single polyphenolic compound, our analysis suggests that flavanols are the major polyphenols in

367 the GSE. With the mass spectrometry and the references characterizing polyphenols in GSE, we

368 chose ten target polyphenolic components, which include three monomers flavan-3-ols (catechin,

369 epicatechin, and epicatechin gallate), four dimers flavan-3-ols (Procyanidin B1, Procyanidin B2,

370 Procyanidin B3, and Procyanidin B4), two phenolic acid (gallic acid and protocatechuic acid), and

371 one stilbene (resveratrol). These ten polyphenolic compounds were further studied through

372 molecular docking simulations.

373

374 **Tulane virus propagation**

375   Tulane virus (TV) was used as a surrogate for human norovirus (HuNoV). Both TV and

376   HuNoV are members of *Caliciviridae* and share structural similarities. TV and HuNoV have a

377   positive-sense, single-stranded genomic RNA of 6.7 kb and 7.5 kb, respectively. About 23-26%

378   of the genome sequences are identical in both viruses (22). The capsid of TV has T=3 icosahedral

379   symmetry and is 40 nm in diameter. The capsid comprises 180 copies of major capsid protein

380   (VP1) or 90 dimers of A/B or C/C. The VP1 is also sub-divided into S, P1, and P2 domains. When

381   the VP1 proteins are assembled, the S domains comprise the bottom surface of the capsid while

382   P1 and P2 domains protrude out of the bottom surface, which is responsible for binding to receptor

383   proteins (**Supplementary Fig. 1**). Similar to HuNoVs, TV recognizes Histo-blood group antigens

384   (23) and sialic acids (24) as cellular receptors. TV is considered more resistant to disinfectants

385   than the other viruses in *Caliciviridae* (48). Therefore, TV has been widely used in virus

386   inactivation experiments as a surrogate for HuNoVs (7, 14, 25–28).

387   TV was a gift from Cincinnati Children's Hospital Medical Center (24) and the TV genome

388   was sequenced as quality control. The genome sequence was 100% identical to those of a wild-

389   type TV strain in Genbank (Access number: EU391643) (7). TV was propagated in MA104 cells,

390   which was purchased from ATCC (CRL-2378.1), and grown in complete culture medium (i.e., 1X

391   minimum essential medium (MEM; Thermo Fisher Scientific, USA), 10% fetal bovine serum

392   (FBS; Thermo Fisher Scientific, USA), 1X antibiotic-antimycotic (Thermo Fisher Scientific,

393   USA), 17 mM of $NaHCO_3$, 10 mM of HEPES, and 1 mM of sodium pyruvate (Thermo Fisher

394   Scientific, USA). When greater than 90% of MA014 cellular monolayers showed cytopathic

395   effects (about 2 days after the inoculation), cells were harvested and collected by centrifugation,

396   and TV was released from host cells by three cycles of freeze and thaw. Virus was separated from

397   cellular debris by centrifugation at 2000 rpm (556 g) for 10 min (Sorvall Legend RT Plus, Thermo

398   Fisher Scientific, USA). The supernatant was treated by filtration with a 0.22 μm bottle top filter

399   (Milliporesigma, USA) to remove additional cellular debris. The virus-containing filtrate was then

400   further purified using an ultracentrifuge (Optima XPN-90 Ultracentrifuge, Beckman Coulter, USA)

401   with a cycle of 1,000 rpm (116 g) for 5 min followed by 36,000 rpm (150700 g) at 4°C for 3 hours.

402   The virus pellet was resuspended in 1X PBS, aliquoted, and stored at -80°C before use.

403

404   **Tulane virus inactivation experiments using GSE**

405   Virus inactivation experiments were initiated by adding 250 μL of TV solution containing 2.5 x10^5

406   PFU TV to 250 μL of GSE-containing solution, in which GSE concentrations ranged from 84 to

407   1694 μg/mL. After the incubation times indicated in the manuscript (i.e., 10 to 120 seconds), 70

408   μL of the mixture was added to 70 μL of FBS to quench the polyphenolic activity (16). Thus, the

409   volumetric ratio of TV, GSE, and FBS in the final solution was 1:1:2. The FBS quenching activity

410   was confirmed in **Supplementary Fig. 5**. Negative controls were prepared for every virus

411   inactivation experiment. In this case, the negative controls were prepared by mixing TV, GSE, and

412   FBS in the ratio of 1:1:2, but in a different mixing order. Specifically, 35 μL of GSE was mixed

413   with 70 μL of FBS to quench the polyphenolic activity followed by adding 35 μL of TV to the

414   mixture (**Fig. 1A**). The final mixture of TV, GSE, and FBS was used for further analysis.

415

416   **Plaque assays**

417   The MA104 cell line was grown in 175 cm^2 flasks (Thermo Fisher Scientific, USA) with the

418   complete culture medium. Cells were seeded into 6-well plates (CC7682-7506, USA Scientific,

419   USA) resulting in cellular monolayers with more than 90% confluency. Cell culture supernatants

420   were aspirated using a vacuum-connected pasteur pipette. 100 μL of TV samples were serially

421   diluted by 10-fold dilutions. Cellular monolayers were incubated with each of the serially-diluted

422   viruses for 1 hour at 37°C with 5% CO_2 to facilitate virus attachment to the MA104 cells. Viruses

423   were aspirated from cellular monolayers and 2 mL of overlay solution containing 1X MEM, 1%

424   agarose, 7.5% sodium bicarbonate, 15 mM HEPES, and 1X antibiotic-antimycotic was added to

425   each well. The overlay solution was solidified at 4°C for 10 minutes. Plates were incubated for 2

426   days at 37°C with 5% CO_2 to allow infectious viruses to form plaques. Next, 2 mL of 10%

427   formaldehyde (VWR, USA) in 1X PBS was added to each well and incubated at room temperature

428   for 1 hour to fix cells. The agarose and the formaldehyde were removed and replaced with a 0.05%

429   crystal violet dye solution. The solution was washed away after 10 minutes, and the number of

430   plaque-forming units (PFU) was counted on a lightbox (ULB-100, Scienceware). The detection

431   limit of the plaque assay was one plaque on the least diluted sample (i.e., 10-fold dilution), which

432   was equivalent to $10^{1.1}$ PFU/mL.

433

434   **Models for virus inactivation kinetics**

435   TV inactivation kinetics were interpreted by a first-order reaction of Chick's law (Eqs. 1-3) and

436   pseudo-second-order model (Eqs. 4-6). Chick's law assumes the activity (i.e., concentration) of

437   available disinfectant remains constant during the reaction. Chick's law has been widely used to

438   describe virus inactivation kinetics by free chlorine, peracetic acid, monochloramine, heat, ozone,

439   and UV (7, 51–54).

440

441

442

$$\frac{dN}{dt} = -kcN \qquad \text{(Eq. 1)}$$

$$\ln\frac{N}{N_0} = -kct \qquad \text{(Eq. 2)}$$

443
$$\log_{10}\frac{N_0}{N} = k'ct \qquad \text{(Eq. 3)}$$

444

445 Where N referred to viral infectivity (PFU/mL), c indicated the TP concentration (µg/mL), t was

446 treatment time (s), k' meant an inactivation rate constant from Chick's law (L/µg·s).

447 The pseudo-second-order model has been widely used to describe reactions where

448 chemisorption between adsorbent and adsorbate is the rate-determining step (29). In this study,

449 TV and TP were considered as adsorbate and adsorbent, respectively.

450

$$\frac{dC_t}{dt} = k_2(C_e - C_t)^2 \qquad \text{(Eq. 4)}$$

$$\frac{t}{C_t} = \frac{1}{C_e^2 k_2} + \frac{1}{C_e}t \qquad \text{(Eq. 5)}$$

451
$$t_{95\%} = \frac{19}{C_e k_2} \qquad \text{(Eq. 6)}$$

452

453 Where t is the reaction time (s), k_2 is the rate constant from the pseudo-second-order model

454 (mL/PFU·s), C_t and C_e are inactivated TV concentration (PFU/mL) at time t and equilibrium state,

455 respectively. Also, $t_{95\%}$ is a reaction time at which C_t reaches 95% of C_e.

456

457

458

Virus particle size

459   The particle size distribution of TV was measured by a light scattering analyzer (DelsaMax

460

461 Pro, Beckman Coulter). TV, GSE, and FBS, either alone or in combination, were prepared in 200

462 μL aliquots, placed in a PMMA cuvette (BrandTech Scientific, USA). Particle size was measured

463 20 times for each sample, and the averaged % intensity was presented with the particle diameter.

464 According to the manufacturer, particle size analysis is reliable within a range from 0.4 to 10,000

465 nm, and all our measurements were in this detection range.

466

467 **Virus aggregation assay**

468 An assay was developed to quantify TV virions that are less than 100 μm in diameter. After the

469 TV inactivation experiments were conducted, the mixture containing viruses, GSE, and FBS was

470 filtered with a 0.1 μm syringe filter (Sartorius, Germany). The number of virus particles in the

471 initial mixture and the filtrate was quantified by one-step RT-qPCR. RNA was extracted from

472 samples using Viral RNA Mini Kit (QIAGEN, Germany) in a final 60 μL volume. RT-qPCR

473 samples were prepared in 96-well plates (4306737, Applied Biosystems, USA) by mixing 5 μL of

474 $2 \times$ iTaq universal SYBR green reaction mix, 0.125 μL of iScript reverse transcriptase from the

475 iTaq universal SYBR green reaction mix (Bio-Rad Laboratories, USA), 3 μL of the RNA, 0.3 μL

476 of 10 μM TV-NSP1-qPCR-F primer, 0.3 μL of 10 μM TV-NSP1-qPCR-R primer, and 1.275 μL

477 of nuclease-free water. The one-step RT-qPCR was run using a qPCR system (QuantStudio 3,

478 Thermo Fisher Scientific, USA) with the following thermocycle: 10 minutes at 50°C and 1 minute

479 at 90°C followed by 40 cycles of 30 seconds at 60°C and 1 minute at 90°C. Detailed information

480 for primers and synthetic DNA controls is summarized in **Table 1**. We obtained a calibration curve

481 by plotting the viral infectivity (Log_{10} PFU/mL) on the x-axis and the outcome of the one step RT-

482   qPCR assay (Log_{10} gc/mL) on the y-axis for the same viral solution (**Supplementary Fig. 6**). The

483   calibration curve was used to determine the reliable range of the aggregation assay. The calibration

484   curve for the aggregation assay was linear (a slope=0.95 and R^2=1.00) between 10^3 and 10^7

485   PFU/mL. The PCR standard curves were obtained with 10-fold serial dilutions of the synthetic

486   DNA oligonucleotide (Integrated DNA technologies, USA) and the PCR efficiency for this one-

487   step RT-qPCR ranged from 85 to 95% (R^2>0.99). Inhibitory effect of GSE was also tested

488   (**Supplementary Fig. 7**). We found 100-fold dilution can reduce the inhibitory effect of GSE to

489   an insignificant level, so the samples were diluted in molecular grade water by 100-fold before the

490   RT-qPCR analysis. Information for RT-qPCR was summarized following the MIQE guidelines

491   (55) in **Supplementary Table 3**.

492   One concern for the aggregation assay was that diluting GSE concentrations and vortexing

493   virus solutions could reverse the aggregation effects induced by GSE, resulting in virus segregation

494   (56–59). Thus, we performed an experiment where TV was inactivated by 847 TP-μg/mL of GSE,

495   which was the highest GSE concentration for the virus aggregation assay (**Fig. 1B**), and

496   subsequently quenched by FBS. Next, samples were serially diluted in complete culture media and

497   vortexed for 10 seconds. These serial dilutions were examined by one-step RT-qPCR to determine

498   the total number of virions regardless of aggregation induced by GSE. Each serial dilution was

499   then subjected to filtration, using filters with a 0.1 μm size pore. The filtrates were also quantified

500   by one-step RT-qPCR to determine the number of virions that passed through the 0.1 μm filter.

501   The differences in the RNA copies after the filtration indicate the number of virion aggregates with

502   a diameter of larger than 0.1 μm after dilution and vortexing. Detailed information on the

503   experiment is elaborated in **Supplementary Text 1**.

504

505   **Table 1.** Information for RT-qPCR conditions and primers

process	Primer name	Sequence[1] (5'-3')	Position in the genome	Amplicon length (bp)	Reaction conditions
RT-qPCR	TV-NSP1-qPCR-F	GTGCGCATCCTTGAGACAAT	879-899	132[2]	50°C for 10 min and 95°C for 1 min, followed by 40 cycles of (95°C for 10 s, 60°C for 30 sec)
	TV-NSP1-qPCR-R	TTGGAGCCGGGTAGAAACAT	991-1011		

506
507   1) Primer pair specificity was checked by the primer-blast tool (National Center of
508   Biotechnology Information). Each pair of primers were blasted with Rhesus monkey
509   (taxid:9544) which was the host organism. As a result, we confirmed that our primers did
510   not target any sequences of the MA104 cells.
511   2) The sequence of standard sample for the NSP gene of Tulane virus (Integrated DNA
512   technologies, USA):
513   5'-
514   AGAATTGGACCGAATTTGGCACACACTCAGAATTTGGTGTGCGCATCCTTGA
515   GACAATAACAGGCACAATACCCCCTTGGAAACCTCACCAGGAATCAATATCT
516   GAAGTTCTGGACGACCTCACACACGGTAAAGTCCAAACAGGTGATGATGTTT
517   CTACCCGGCTCCAAAGGTTGAGCGACACTATCAAAGATCTGAGTGTCATGGC
518   TTGTGATCCCTCTGCACCGCCCGAAGTTGCGC-3' - (GenBank accession number:
519   EU391643).
520

Molecular docking simulation for polyphenols and capsid proteins interaction

521

522   We selected ten polyphenolic compounds that are present in GSE, including two phenolic

523   acids, three monomer flavan-3-ols, and four dimer flavan-3-ols (37). The structural information

524   on the polyphenols were obtained from PUBChem (https://pubchem.ncbi.nlm.nih.gov/). Although

525   we conducted inactivation experiments with Tulane virus, we obtained HuNoV capsid protein

526   instead of TV from the Protein Data Bank (PDB, https://www.rcsb.org/) to study interactions with

527   the polyphenols because the database of PDB does not provide TV capsid information. Cryo-EM

528   structure analysis showed that the TV capsid structure closely resembles that of HuNoV(32).

529   We downloaded the HuNoV icosahedral asymmetric unit (PDB ID: 1IHM) which is a basic

530   building block for the HuNoV capsid. This icosahedral unit comprised three VP1 proteins (i.e.,

531   chain A, B, and C in PDB format). The complete HuNoV capsid consists of 180 identical

532   icosahedral asymmetric units. We also used a deep-learning-based structure prediction tool -

533   trRosetta(60), to predict the 3D-structure of the TV capsid (**Supplementary File 1**) from its

534   sequence and a multiple-sequence alignment of related sequences. We studied molecular docking

535   of the polyphenols to HuNoV and to other different enteric viruses including hepatitis A virus

536   (PDB: 4QPI), murine norovirus-1 (PDB: 6S6L), and feline calicivirus-F9 (PDB: 6GSH). Bovine

537   serum albumin (BSA) is a major component for FBS, and BSA showed a strong binding affinity

538   to the polyphenols as confirmed by the quenching effect in the virus inactivation experiments.

539   BSA (PDB: 4F5S) was used as a positive control for molecular docking with the polyphenolic

540   compounds. Next, we used the target proteins (capsids or BSA) and flexible conformations of all

541   the aforementioned ten polyphenol ligands to discern stable docking conformations, record

542   binding affinity scores, and report across four capsid domains where each of the polyphenols tend

543   to bind in order of domain preference. The flexible docking protocol is similar to the Z-Dock

544   protocol (38) as implemented within OptMAVEn-2.0(39).

545

546   **Statistical analysis**

547   All experiments were repeated three times with distinct virus samples (i.e., three biological

548   replications), each of which was analyzed by three separate RT-qPCR measurements (i.e., three

549   technical replications). Paired sample t-test was used to compare results of plaque assay and

550   aggregation assay in **Fig. 2C** and binding energies of different viral species (HuNoV, FCV, MNV,

551   and HAV) to polyphenolic compounds in **Fig. 6**. We confirmed that all data for statistical analysis

552   satisfied assumptions of paired sample t-test (i.e., no outliers and normality). For example, all data

553   for statistical analysis were between Q1-1.5IQR and Q3+1.5IQR range, which means there were

554   no outliers. Also, differences between two data sets (e.g., plaque assay versus aggregation assay

555   in **Fig. 2C** or HuNoV versus MNV or HAV in **Fig. 6**) were normally distributed (P>0.05).

556

557   **Acknowledgement**

561

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728

730 **Fig. 1. Inactivation effects of GSE on Tulane virus.** 10^6 PFU Tulane virus was incubated with
731 the indicated amount of GSE. FBS was added to quench each reaction at the indicated times. Initial
732 virus titer (PFU_0) was divided by the virus titer at each indicated time (PFU). Thus, the y-axis
733 (Log_{10} PFU_0/PFU) indicates the decrease in virus titer caused by GSE on log_{10} scale. For all
734 experiments, each symbol represents one molecular replicate. **(A)** The dashed lines were trend
735 lines calculated by the pseudo-second-order model. TV inactivation as a function of GSE
736 concentration and time. **(B)** TV inactivation as a function of GSE concentration. In this experiment
737 10^6 PFU TV was incubated for 120 seconds with the indicated GSE concentrations. **(C)** TV
738 inactivation as a function of initial TV titer. Varying amounts of TV (indicated on the x-axis) were
739 added to reactions containing 42 μg/mL GSE. All reactions were quenched with FBS after a 120
740 second incubation. **(D)** A comparison of GSE versus peracetic acid and free chlorine inactivation.
741 10^5 or 10^6 PFU TV was incubated with GSE (42 μg/mL) for 120 sec. The trend lines for GSE were
742 determined by the pseudo-second-order model. The arrow indicates a detection limit. Trend lines
743 for 42 μg /mL free chlorine or peracetic acid were derived by Chick's law with the rate constants
744 determined by our previous studies (7, 14).
745
746 **Fig. 2. GSE-induced TV aggregation. (A)** Solutions either lacking (control) or containing 10^6
747 PFU TV were each incubated with 847 μg/mL of GSE followed by ultracentrifugation (150700 g)
748 for 3 hours. GSE concentrations were quantified and statistical analysis was performed using the
749 Mann-Whitney test (P<0.05) **(B)** The particle size distribution of TV in the presence or absence of
750 GSE. **(C)** A dose-response curve for plaque and aggregation assays. Plaque assay results were
751 normalized to the initial virus titer and presented in Log_{10} PFU_0/PFU to indicate the decrease in
752 virus titer with different total polyphenol concentrations. Aggregation assay was analyzed by the
753 one-step RT-qPCR and presented in the normalized gene copy number (Log_{10} gc_0/gc) to show the
754 reduction in the number of virions. Equivalent reductions in PFU or gc (%) were also presented
755 on the right y-axis for plaque assay and aggregation assay, respectively. For these experiments,
756 10^6 PFU TV was incubated with varying concentrations of GSE for 2 minutes followed by
757 quenching the reaction with FBS. Each symbol indicates one separate experiment where one
758 plaque assay or three qPCR analyses were conducted.
759

760 **Fig. 3.** Summary of molecular docking analysis between HuNoV capsid and selected polyphenols
761 found in GSE. BSA, which is known to bind strongly with polyphenols (16), was used as a positive
762 control. The expected binding strength of each polyphenol (in increasing order of size/molecular
763 weight) with HuNoV capsid protein (PDB id: 1IHM) has been reported where the error bars
764 indicate variance across top 15 polyphenol binding poses within the same capsid groove. The scale
765 on the right indicates fractional capsid-binding affinity of a given polyphenol with respect to the
766 positive control – BSA.
767

768 **Fig. 4.** Larger polyphenols show greater electrostatic stabilization with the viral capsid. **(A)**
769 HuNoV capsid shows a trigonal-symmetric polyphenol accessible groove (indicated in teal), which
770 includes three intra-chain pockets, and three inter-chain binding crevices. Smaller polyphenols can
771 access the inter-chain crevices, while the bigger ones remain localized to the more solvent-exposed

772   intra-chain pockets. **(B)** Residue level interactions of Procyanidin-B1 with the HuNoV capsid are
773   listed. **(C)** A graphical overview of the exact docked pose of Procyanidin-B1 in the capsid groove
774   with most of the interactions is illustrated.
775
776   **Fig. 5**. All polyphenol binding is primarily localized around the S and S-P1 hinge domains of the
777   HuNoV capsid - while the P2 domain remains mostly unbound. Smaller polyphenols can access
778   more buried inter-chain pockets of the HuNoV capsid, while larger Procyanidins bind to surface-
779   exposed pockets with a single chain. Pockets guarded by each chain have been indicated by a
780   different color (green – A, cyan – B, magenta – C). Smaller polyphenols – protocatechuate, gallate,
781   and catechin bind best at the interface of chains B and C. Slightly larger epicatechin and
782   epicatechin gallate bind best at the interface of chain A and B, while larger procyanidins (all B1
783   through B4) bind best to a solvent-exposed cavity within chain A only.
784
785   **Fig. 6.** Expected binding energies (absolute modal values) between each polyphenolic compound
786   and the four viral capsids (HuNoV: human norovirus, FCV: feline calicivirus F9, MNV: murine
787   norovirus strain 1, and HAV: hepatitis A virus) were computed using the Rosetta binding energy
788   function from top 15 docking poses per complex. The error bars indicate variance from the 15
789   docked poses for each complex. The reported energy scores were compared by a paired sample t-
790   test indicating a statistically significant higher polyphenol binding activity by HuNoV and FCV in
791   contrast to MNV ($P < 0.05$) and HAV ($p < 0.001$) capsids.
792