Comparison of antioxidant activity between cyanidin-3-O-glucoside (C3G) liposome and cyanidin-3-O-glucoside (C3G) in 2D and 3D cell cultures

Tisong Liang^{1,†}, Rongfa Guan^{1,*†}, Guozhou Cao², Haitao Shen³, Zhenfeng Liu⁴, Qile Xia⁵, Zhe Wang¹

¹ Zhejiang Provincial Key Laboratory of Biometrology and Inspection and Quarantine, China Jiliang University, Hangzhou 310018, China;

² Ningbo Academy of Inspection and Quarantine, ningbo yingyi road No.66 A building room 518, nignbo 315012,China;

³ Zhejiang Provincial Center for Disease Control and Prevention, 3399 Binsheng Road, Hangzhou 310051, China;

⁴ Chiatai Qingchunbao Pharmaceutical Co., LTD , NO. 551 Xixi Road, Hangzhou 310023, China;

⁵ Food Science Institute, Zhejiang Academy of Agricultural Sciences, 298 Desheng Road, Hangzhou 310021, China;

* Correspondence: Rongfaguan@163.com; Tel.: +86-571-876-76269; Fax: +86-571-869-1449

† These authors contributed equally to this work.

42

43 **ABSTRACT**

44 The 2D cell culture is the predominant in vitro model for numerous studies.
 45 However, 2D cell cultures may not accurately reflect the functions of
 46 three-dimensional (3D) tissues, which have extensive cell–cell and cell–matrix
 47 interactions; thus, using 2D cell cultures may lead to inaccurate experimental results.
 48 Therefore, to obtain adequate and detailed information about the antioxidant activity
 49 of cyanidin-3-O-glucoside (C3G) and C3G liposomes in the 2D and 3D cell culture
 50 models, we used in this study H₂O₂ to construct the cell damage model and assess the
 51 antioxidant activity of C3G and C3G liposomes on Caco-2 cells cultured in the 3D
 52 model. We also measured the cell viability, cell morphology, and activity of
 53 glutathione (GSH), superoxide dismutase (SOD), total antioxidant capacity (T-AOC),
 54 and malondialdehyde (MDA) content of Caco-2 cells treated with H₂O₂, C3G, and
 55 C3G liposomes. Results showed that cells cultured in the 3D culture model formed a
 56 3D structure and tight spheroids and showed increased cell activity and IC₅₀. The C3G
 57 and C3G liposomes can enhance the activity of GSH, SOD, and T-AOC but decrease
 58 the MDA content. At the same time, the effect was more obvious in the 3D cell
 59 culture model than in the cells cultured in the 2D model. This study revealed that the
 60 results obtained from the 2D cell model may be inaccurate compared with the results
 61 obtained from the 3D cell model. A realistic mechanism study of antioxidant activity
 62 of C3G and C3G liposomes in the 3D cell model, which acts as an intermediate stage
 63 bridging the in vitro 2D and in vivo models, was observed.

64 **Keywords:** 3D cell culture; C3G; C3G liposomes; Antioxidant activity; Caco-2 cell

65 1. Introduction

66 Anthocyanins are water-soluble vacuolar pigments found in most species in the
67 plant kingdom and are responsible for the red, purple, and blue colors of many fruits,
68 vegetables, and flowers^{1, 2}. Anthocyanins exhibit potential free radical-scavenging
69 activities that prevent low-density lipoprotein oxidation and positively affect chronic
70 gut inflammatory diseases, obesity, and inflammation³⁻⁵. Moreover,
71 cyanidin-3-O-glucoside (C3G), which is an anthocyanin with a high percentage and
72 belongs to the flavonoid family and commonly present in human diet, exhibits
73 antiinflammatory and antioxidant effects⁶⁻⁸. Some studies have showed that C3G
74 protects from the adverse effects of radiation, controls the key aspects of
75 tumorigenesis, inhibits proliferation, induces apoptosis of cancer cells, reduces
76 oxidative stress, fights H₂O₂-induced oxidative stress in human embryonic kidney
77 cells, induces cell apoptosis and inhibit cell migration⁹⁻¹⁵.

78 Liposomes are vesicles, wherein the small aqueous volumes are surrounded by
79 bilayer membranes that are normally composed of phospholipids^{16, 17}. Liposomes can
80 enhance the stability and bioavailability of encapsulated materials by protecting them
81 from external environment factors, making them ideal candidates for drug delivery
82 and important in food systems¹⁸⁻²¹.

83 The two-dimensional (2D) monolayer cell models have low anatomical and
84 physiological relevance and remain the predominant in vitro model for numerous
85 studies^{22, 23}. However, some disadvantages exist in the use of the 2D monolayer cell
86 model in vitro in some studies. Growing concerns have been demonstrating that

monolayer and monotypic (2D) cellular screening assays may not effectively reproduce the response of a three-dimensional (3D) solid tumor to pharmacological compounds^{24, 25}. In cancer research, the limitations of the 2D models are considered one major reason for the approximately 95% of potential anticancer drugs failures in clinical trials²⁶.

The 3D spheroid cell culture systems are providing new insights into tumor biology and also in differentiation, tissue organization, and homeostasis^{27, 28}. Cells in in vitro 3D culture systems are in league with their counterparts in vivo compared with cells grown as 2D monolayers, wherein 2D monolayer cells also exhibit altered cell cycle durations, morphologies, susceptibility to drugs, metabolism, and gene and protein expression levels^{29, 30}. Cells cultured as 3D models exhibit features that are close to the complex in vivo conditions³¹ and have been proven realistic for translating the study findings for in vivo applications; moreover, culturing cell lines as 3D models induces them to behave a step closer to the natural conditions^{32, 33}. The 3D models also can help investigate the interplay between different physiological conditions (oxygen or nutrient deprivation), irradiation, or other physical and chemical stimuli³⁴. We used liposome technology because liposomes can protect embedding materials and thus improve the effectiveness and stability of C3G, which could be used as a carrier.

Therefore, the objectives of this study were to compare the antioxidant activity of C3G and C3G liposomes by assessing the cellular viability in Caco-2 cells cultured in 2D and 3D conditions. We compared the antioxidant activity via the assay of

109 glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA), and total
110 antioxidant capacity (T-AOC).

111 **2.Materials and methods**

112 **2.1Materials**

113 The Caco-2 cells (CBCAS, Shanghai, China) , Cyanidin-3-glucoside (C3G) was
114 purchased from Chengdu Biopurify Phytochemicals Ltd (Chengdu,China).
115 Phosphatidylcholine (PC) and cholesterol (CH) were purchased from Beijing
116 Shuangxuan Microorganism Co. Ltd (Beijing, China). Chloroform and diethyl ether
117 were obtained from Hangzhou Jiachen Chemical Company (Hangzhou, China). All
118 other chemicals were of reagent grade. The water used for all experiments was
119 deionized water.

120 **2.2 Methods**

121 **2.2.1 Preparation of C3G liposomes**

122 C3G liposomes were prepared by reverse-phase evaporation method^{35, 36}.
123 Phosphatidylcholine and cholesterol were dissolved in chloroform-diethyl ether(W_{PC} :
124 W_{CH} =2.87:1). C3G was dissolved in phosphate-buffered saline (PBS) (0.20 M, pH
125 7.4). Then, the organic phase was homogenized with the aqueous phase by probe
126 sonication for 10 min. The mixture was transferred to a round-bottomed flask. The
127 organic solvent was evaporated under reduced pressure with a rotary evaporator to
128 form a gel. Subsequently, 30 mL of phosphate-buffered solution was added to the gel,
129 which was then probe-sonicated for an additional 25 min. The as-prepared liposomes
130 were stored at 4 °C for further study.

131 **2.2.2 Morphology of the liposomes**

132 Transmission electron microscopy (JEM-2100, Japanese Electronics Co., Ltd.,
133 Tokyo, Japan) was employed to determine the microstructure of C3G liposomes via
134 negative staining method. A drop of this solution was placed on a
135 Formvar-carbon-coated copper grid for 5 min and was then imaged³⁷.

136 **2.2.3 Cell culture**

137 **2.2.3.1 Cell culture**

138 The Caco-2 cells (CBCAS, Shanghai, China) were maintained in minimum
139 essential medium (MEM) (Hyclone Laboratories, Inc., USA) supplemented with 10%
140 fetal bovine serum (FBS) Hyclone Laboratories, Inc., USA, penicillin (100 kU/L), and
141 streptomycin (100 g/L) at 37 °C under a 5% CO₂ atmosphere in a humidified
142 incubator³⁸⁻⁴⁰. After reaching 70%–80% confluence, the cells were subcultured and
143 maintained with medium changes every 2–3 days.

144 **2.2.3.2 2D cell culture**

145 Caco-2 cells were isolated using 0.25% Trypsin-EDTA (Sigma, USA) and
146 centrifuged at 1000 rpm for 5 min. Isolated cells were seeded at 24- and 96-well
147 plates according to the experimental design. The cells were then cultured using MEM
148 supplemented with 10% FBS (Gibco BRL Co., Ltd., USA) and 1% antibiotics (100
149 IU/mL penicillin, and 100 µg/mL streptomycin) and maintained at 37 °C in an
150 atmosphere of 95% air with 5% CO₂. All the experiments were performed on
151 logarithmically growing cells. Prepared cells were exposed to various C3G liposome
152 concentrations (0, 0.05, 0.10, 0.15, 0.20, or 0.25 mg/mL).

153 **2.2.3.3 3D cell culture**

154 The 3D cell culture was carried out using the 3D cell culture hydrogel kit
155 (Hangzhou Kevin Biotechnology Co., Ltd., Hangzhou, China) in accordance with the
156 manufacturer's directions. The Caco-2 cells were isolated as previously described,
157 and the cell suspension was mixed with the gel (vol:vol=1:1) and shaken slightly and
158 rapidly on a vortex for 1 s and repeated 1–2 times. Subsequently, the gel–cell mixture
159 was added to the well and incubated in the plate in an incubator at 37 °C for 5–10 min.
160 Lastly, the culture medium was added to the plate, and the plate was moved to the
161 humidified incubator and maintained at 37 °C in an atmosphere with 5% CO₂. The
162 medium was replaced every 1–2 days. All the experiments were performed on
163 logarithmically growing cells.

164 **2.2.4 Cell morphology**

165 For documenting the morphology of Caco-2 cells in 3D culture condition, the
166 cells were washed with PBS twice. Phase contrast images of the cells cultured in 3D
167 conditions were observed using an inverted microscope (Nikon Eclipse Ti, Nikon,
168 Tokyo, Japan) at different magnifications⁴¹.

199 Scanning electron microscopy was used to analyze the cell morphology in different
200 cell culture models. The Caco-2 cells (1×10^5) were seeded on glass slides placed in a
201 6-well plate for 2 days. Then, the samples were removed from culture wells, rinsed
202 with PBS, and fixed with 2.5% glutaraldehyde for 8 h at 4 °C. Subsequently, the
203 samples were rinsed again and postfixed in osmium tetroxide for 1 h before being
204 dehydrated in a series of graded ethanol solutions (30%, 50%, 70%, 80%, 90%, and

95% vol/vol). Final drying was performed by critical-point method. Finally, gold-coated specimens were examined by SEM (Hitachi, SU-8010, Tokyo, Japan)⁴².

2.2.5 Cell viability

Cell viability was determined by 2,5-diphenyl tetrazolium bromide (MTT) assay at 550 nm with a microplate reader (Tecan Co., Weymouth, UK)⁴³. The cells were seeded in 96-well plates in a 100 µL volume (1×10^4 per well) and allowed to grow for 24 h before treatment with different concentrations of C3G liposomes for 24 h. At the end of the experiments, 10 µL of 5 µg/mL MTT was added to each well. The cells were then incubated at 37 °C for 4 h. Formazan was solubilized in 150 µL of dimethyl sulfoxide and measured at 550 nm. The results were given as relative values to the negative control in percentage, whereas the positive control was set to be 100% viable. The percentage of cell proliferation was calculated as⁴⁴:

$$\text{Cell viability}(\%) = \frac{A_{exp} - A_{neg}}{A_{con} - A_{neg}},$$

where A_{exp} is the amount of experimental group absorbance, A_{neg} is the amount of blank group absorbance, and A_{con} is the amount of the control group absorbance.

2.2.6 Assay of GSH and SOD activity

The assay of GSH and SOD activities was detected by glutathione peroxidase assay kit and total superoxide dismutase assay kit according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, China). The Caco-2 cells were cultured in a 25 cm² culture flask and exposed to C3G and C3G liposomes for 12 h. The cells were then collected through centrifugation at 400 ×g for 5 min at 4 °C and the supernatant was removed. The cell suspension was washed and centrifuged twice

227 using ice-cold PBS to remove all traces of the medium. The cell pellet was sonicated
228 at 300 W for 10 s (three cycles) to obtain lysates, which were then centrifuged at
229 12,000 rpm for 10 min. Cell supernatants were finally assayed to measure GSH and
230 SOD activities^{45, 46}.

231 Exactly 600 μ L of the cell suspension, 150 μ L of the substrate solution, and 600
232 μ L of the reaction buffer solution were transferred to a fresh tube. The standard group
233 included 25 μ mol/L GSH dissolved in a GSH buffer solution. In the blank group,
234 GSH was replaced by PBS. A microplate reader estimated the absorbance at 405 nm,
235 and protein content was measured using the Bradford method using bovine serum
236 albumin as the standard.

237 Exactly 20 μ L of the prepared sample, 200 μ L of the SOD working solution, 20
238 μ L of the enzyme working solution, and 20 μ L of dilution buffer were added to each
239 well of a 96-well plate, and the mixture was mixed thoroughly. The plate was
240 incubated for 20 min at 37 °C, and a plate reader was used to detect the absorbance at
241 450 nm.

242 **2.2.7 T-AOC assay and MDA assay**

243 The Caco-2 cells were cultured in a 25 cm² culture flask and exposed to C3G and
244 C3G liposomes for 12 h and collected for the T-AOC and MDA level tests. The
245 T-AOC and MDA levels in Caco-2 cells were measured with T-AOC and MDA
246 analysis kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following
247 the manufacturer's protocols^{47, 48}.

248 The Caco-2 cells were cultured in a 25 cm² culture flask and exposed to C3G and

249 C3G liposomes for 12 h. The cells were then collected through centrifugation at 400
250 $\times g$ for 5 min at 4 °C and the supernatant was removed. The cell suspension was
251 washed and centrifuged twice using ice-cold PBS to remove all traces of the medium.
252 The cell pellet was sonicated at 300 W for 10 s (three cycles) to obtain lysates, which
253 were then centrifuged at 12,000 rpm for 10 min. Cell supernatants were finally
254 assayed to measure the assay of T-AOC and MDA.

255 For the detected of T-AOC of cells, exactly 100 μ L of the cell suspension added
256 the reagent and the samples were treated according the manufacturer's protocols. A
257 microplate reader estimated the absorbance at 520 nm, and protein content was
258 measured using the Bradford method using bovine serum albumin as the standard.

259 For the detected of MDA, exactly 200 μ L of the cell suspension added the reagent
260 and the samples were treated according the manufacturer's protocols. The plate was
261 incubated for 20 min at 37 °C, and a plate reader was used to detect the absorbance at
262 532nm.

263 **2.2.8 Statistical analysis**

264 All experiments were performed thrice in duplicate per sample. Data are
265 presented as the means $\pm$ standard deviations from at least three independent
266 measurements. Statistical analysis was performed using SPSS version 21.0 for
267 Windows.

268 **3 Results**

269 **3.1 Characterization studies**

270 Transmission electron microscopy (TEM) was used to investigate the

271 morphology of C3G liposomes. Figure 1 shows a recorded representative TEM image
272 of C3G liposomes. The nanoparticles exhibited spherical shapes, and the size of C3G
273 liposomes was approximately 200 nm and formed a vesicular structure. From our
274 previous study³⁶, the average diameter of C3G liposomes is 165.78 ± 4.3 nm, the
275 encapsulation efficiency of C3G liposomes is $70.43\% \pm 1.95\%$.

276 **3.2 Cell morphology**

277 In our work, Caco-2 cells were cultured in the 3D models for 7 days and washed
278 with PBS thrice. Subsequently, cell morphology was observed at different
279 magnifications using an inverted microscope and scanning electron microscope.

280 As shown in Fig.2 and Fig.3, the morphology of the cell culture in the 3D model
281 was different from the cell culture in the 2D model. From Fig.2, at different
282 magnifications, the cells showed a mass-like spheroid growth, and the cells gathered
283 and grew together. From the scanning electron microscope image, the Caco-2 cells
284 grew together in patches in the 2D culture. However, when grown in the 3D cell
285 culture model, they formed uniform spheroids with relatively smooth surfaces. The
286 SEM image showed that the Caco-2 cell culture in the 3D culture model formed a 3D
287 structure and tight spheroids.

288 **3.3 Cell viability**

289 In this study, Caco-2 cells cultured in 2D and 3D models were treated with C3G
290 and C3G liposomes. Then, the cell viability was detected by MTT assay. The result is
291 shown in Fig.4.

292 As shown in Fig. 4, the MTT assay results demonstrated a
293 concentration-dependent activity after exposure to C3G and C3G liposomes in
294 different cell culture models. From Fig. 4A, the cells in different culture models

295 exhibit different cell activities after being exposed to C3G. In the 3D culture model,
 296 the cell activity is higher than that cultured in the 2D culture model. IC₅₀ of cells
 297 exposed to C3G in the 2D and 3D cell culture models are 0.19 and 0.25mg/mL, and
 298 0.175 and 0.233 mg/mL when exposed to C3G liposomes in different culture models,
 299 respectively. Figure 4B shows that the activity of cells exposed to C3G liposomes and
 300 cells cultured in the 3D culture model is higher than the cells cultured in the 2D
 301 culture model. Cells cultured in the 3D culture model show higher cell activity and
 302 IC₅₀ compared with the 2D cell culture model in this study. This phenomenon is due
 303 to multicellular resistance and the adhesion between cells, thereby reducing the
 304 sensibility of Caco-2 cells to the C3G and C3G liposomes^{49, 50}. At the same time,
 305 collagen gels purported to represent well in vivo conditions possibly causes the strong
 306 increase of cell viability.

307 **3.4 Assay of GSH activity in different cell culture models**

308 To compare the difference of GSH activity in different cell culture models, the
 309 Caco-2 cells cultured in the 2D and 3D culture models were treated with
 310 H₂O₂(0.2mmol) ,C3G, and C3G liposomes for 12 h, and the GSH activity was tested.
 311 Figure 5 shows the GSH activity in the 2D and 3D cell models. From Fig. 5, the GSH
 312 activity in different cell culture models is significantly different ($p<0.05$). The C3G
 313 and C3G liposomes both can enhance the GSH activity treated with H₂O₂, and the
 314 enhancement in the 3D model is higher than that in the 2D model. At the same time,
 315 the enhancement of C3G liposome is higher than that of C3G. This phenomenon is
 316 because the liposomes protect the C3G from the effect of external environment factors,
 317 leading to the high effectiveness of cells and multicellular resistance, and the adhesion
 318 between cells reduces the sensibility of Caco-2 cells to the C3G and C3G liposomes^{49,}
 319 ⁵⁰.

320 **3.5 Assay of SOD activity in different cell culture models**

321 To compare the difference of SOD activity in the 2D and 3D culture models, the
 322 Caco-2 cells cultured in the 2D and 3D culture models were treated with
 323 H₂O₂(0.2mmol), C3G, and C3G liposomes for 12 h, and the SOD activity was tested.
 324 Fig. 6 shows the SOD activity in the 2D and 3D cell models, and the SOD activity in
 325 different cell culture models is significantly different ($p<0.05$). The C3G and C3G
 326 liposomes both can enhance the SOD activity treated with H₂O₂, and the enhancement
 327 in the 3D model is higher than that in the 2D model. At the same time, the
 328 enhancement of C3G liposome is higher than that in C3G.

329 **3.6 Assay of MDA in different cell culture models**

330 To compare the difference of MDA content in different cell culture models, the
 331 Caco-2 cells in different culture models were treated with H₂O₂(0.2mmol), C3G, and
 332 C3G liposomes for 12 h, and the GSH activity was tested. Figure 7 shows the MDA
 333 content in the 2D and 3D cell models, and the result indicated that H₂O₂ can increase
 334 the MDA content after treatment. The C3G and C3G liposomes both can decrease the
 335 MDA content in different cell culture models. As for the reduced effect, the C3G
 336 liposomes are higher than C3G. The result showed that C3G and C3G liposomes both
 337 can decrease the MDA content in different cell culture models and the reduced effect.
 338 The C3G liposomes are higher than C3G. This phenomenon is because the liposomes
 339 protect the C3G from the effect of external environment factors, leading to the high
 340 effectiveness of cells in different culture models.

341 **3.7 Assay of T-AOC in different cell culture models**

342 To compare the difference of T-AOC in different cell culture models, the Caco-2
343 cells cultured in the 2D and 3D culture models were treated with H₂O₂(0.2mmol),
344 C3G, and C3G liposomes for 12 h, and T-AOC assay was tested. Figure 8 shows the
345 T-AOC assay in the 2D and 3D cell models. From Fig. 8, the T-AOC assay in
346 different cell culture models is significantly different ($p<0.05$). The C3G and C3G
347 liposomes both can enhance the assay of T-AOC treated with H₂O₂, and the
348 enhancement in the 3D model is higher than that in the 2D model. At the same time,
349 the enhancement of C3G liposome is higher than that of C3G.

350 The result indicated that the C3G and C3G liposomes can enhance the T-AOC
351 assay and then play an antioxidant role in Caco-2 cells.

352 **4.DISCUSSION**

353 In this study, we compared the antioxidant activity of C3G and C3G liposomes in
354 different cell culture models. The cell were treat with H₂O₂ and incubated with the
355 C3G and C3G liposomes. The result indicated that : the C3G and C3G liposomes can
356 increase the concentration of GSH, improve the activity of SOD and T-AOC ,the
357 content of MDA were decreased after treated. In the 3D cell culture model, the effect
358 is more obvious than cells cultured in 2D model.

359 C3G is a potent antioxidant that displays anticancer properties in vitro and in
360 vivo^{51,52}. However, few studies have evaluated the antioxidant properties of C3G and
361 C3G liposomes in the 3D cell culture model. The results of this study revealed that
362 C3G and C3G liposomes can enhance the activity of GSH, SOD, and T-AOC and
363 decrease the MDA content in Caco-2 cells in certain concentrations. However, this

work did not focus on the molecular level of C3G and C3G liposomes to study the antioxidant activity of Caco-2 cells. Several studies have investigated the antioxidant activity of C3G on cells at the molecular level. Thus, further studies on the antioxidant activity of C3G liposomes on cells at the molecular level are needed.

At the same time, the 3D cell culture model was developed as a new in vitro model. The cells cultured in 3D model exhibit features that are close to the complex in vivo conditions³¹ and have been proven realistic for translating the study findings for in vivo applications. In our work, we used hydrogel and built the 3D cell culture model. This method is inconvenient and time consuming. Thus, in future studies, we will use other materials (type I collagen matrix, 3D scaffolds) to construct the 3D cell culture model and compare the differences in different 3D cell culture models. Meanwhile, as for the C3G liposome, a small size of C3G liposomes is expected, making them more effective than C3G. At the same time, the uniform particle size is needed, which can improve the stability of liposomes, because the liposomes were used as carriers, the size is smaller, and they are more effective. The new method, which is uninjurious for liposome preparation, is important in our further studies.

Therefore, we need a new 3D cell culture model to explore the antioxidant mechanisms of C3G and C3G liposomes at the molecular level in the 3D cell culture model. Meanwhile, the properties, expression of specific genes, and protein effects, and mechanisms of C3G liposomes on cells will be explored in further studies, another cells will used to build the 3D cell culture model and study the properties of C3G liposomes in future study is needed.

386 **5. CONCLUSIONS**

387 In summary, the mechanism of C3G and C3G liposomes on cells in different cell
388 culture models are as follows. The C3G liposomes and C3G can inhibit cell
389 proliferation. The inhibiting effect of C3G liposomes is higher than that of C3G. In
390 the 3D cell culture model, the inhibiting effect is lower than that in the 2D cell culture
391 model because in 3D conditions, the multicellular resistance and the adhesion
392 between cells reduce the sensibility of Caco-2 cells to the C3G and C3G liposomes.
393 Assay of GSH, SOD, MDA, and T-AOC demonstrated that the C3G and C3G
394 liposomes can enhance the activities of GSH, SOD, and T-AOC and decrease the
395 MDA content in different cell culture models. At the same time, in the 3D cell culture
396 model, these results are more obvious than that in the 2D cell culture model.

397 **Competing interests**

398 The Author(s) declare(s) that they have no conflicts of interest to disclose.

399 **Acknowledgements**

400 This work was supported by Zhejiang Provincial Key Laboratory of Biometrology
401 and Inspection and Quarantine , and National & Local United Engineering Lab of
402 Quality Controlling Technology and Instrumentation for Marine Food. We gratefully
403 acknowledge financial support from National Natural Science Foundation of China
404 (31571845), National Key R&D Program of China (grant No.2016YFD0401503),
405 The Key Research and Development Program of Zhejiang province (2018C02049).

406 **Abbreviations**

407 C3G Cyanidin-3-O-glucoside

408 Caco-2 cells Human epithelial colorectal adenocarcinoma cells

409	MEM	Minimum essential medium
410	FBS	Fetal Bovine Serum
411	PC	Phosphatidylcholine
412	CH	Cholesterol
413	TEM	Transmission electron microscope
414	SEM	Scanning electron microscope
415	2D	Two-dimensional
416	3D	Three-dimensional
417		
418		
419		
420		
421		
422		
423		
424		
425		
426		
427		
428		
429		
430		

431

432

433

434

435 References

436

- 437 1. P. Jing, B. Qian, S. Zhao, X. Qi, L. Ye, M. Monica Giusti and X. Wang, Effect of glycosylation
438 patterns of Chinese eggplant anthocyanins and other derivatives on antioxidant effectiveness
439 in human colon cell lines, *Food Chem*, 2015, **172**, 183-189.
- 440 2. C. Tan, G. B. Celli and A. Abbaspourrad, Copigment-polyelectrolyte complexes (PECs)
441 composite systems for anthocyanin stabilization, *Food Hydrocolloids*, 2018, **81**, 371-379.
- 442 3. L. Yi, C.-y. Chen, X. Jin, M.-t. Mi, B. Yu, H. Chang, W.-h. Ling and T. Zhang, Structural
443 requirements of anthocyanins in relation to inhibition of endothelial injury induced by
444 oxidized low-density lipoprotein and correlation with radical scavenging activity, *FEBS letters*,
445 2010, **584**, 583-590.
- 446 4. R. Cuevas, iacute, E. O. guez, V. P. Dia, G. G. Yousef, P. A. Garcia-Saucedo and J. Lopez-Medina,
447 Inhibition of pro-inflammatory responses and antioxidant capacity of Mexican blackberry
448 (*Rubus* spp.) extracts, *Journal of agricultural and food chemistry*, 2010, **58**, 9542-9548.
- 449 5. A. Sousa, P. Araújo, J. Azevedo, L. Cruz, I. Fernandes, N. Mateus and V. de Freitas, Antioxidant
450 and antiproliferative properties of 3-deoxyanthocyanidins, *Food Chem*, 2016, **192**, 142-148.
- 451 6. H. Jung, H.-K. Kwak and K. T. Hwang, Antioxidant and antiinflammatory activities of
452 cyanidin-3-glucoside and cyanidin-3-rutinoside in hydrogen peroxide and
453 lipopolysaccharide-treated RAW264.7 cells, *Food Science and Biotechnology*, 2014, **23**,
454 2053-2062.
- 455 7. M.-M. Ma, Y. Li, X.-Y. Liu, W.-W. Zhu, X. Ren, G.-Q. Kong, X. Huang, L.-P. Wang, L.-Q. Luo and
456 X.-Z. Wang, Cyanidin-3-O-Glucoside Ameliorates Lipopolysaccharide-Induced Injury Both In
457 Vivo and In Vitro Suppression of NF- κ B and MAPK Pathways, *Inflammation*, 2015, **38**,
458 1669-1682.
- 459 8. H. Cai, B. Yang, Z. Xu, B. Zhang, B. Xu, X. Li, P. Wu, K. Chen, R. V. Rajotte, Y. Wu and G. R. Rayat,
460 Cyanidin-3-O-Glucoside Enhanced the Function of Syngeneic Mouse Islets Transplanted
461 Under the Kidney Capsule or Into the Portal Vein, *Transplantation*, 2015, **99**, 508-514.
- 462 9. P. Pratheeshkumar, Y. O. Son, X. Wang, S. P. Divya, B. Joseph, J. A. Hitron, L. Wang, D. Kim, Y.
463 Yin, R. V. Roy, J. Lu, Z. Zhang, Y. Wang and X. Shi, Cyanidin-3-glucoside inhibits UVB-induced
464 oxidative damage and inflammation by regulating MAP kinase and NF-kappaB signaling
465 pathways in SKH-1 hairless mice skin, *Toxicology and applied pharmacology*, 2014, **280**,
466 127-137.
- 467 10. P. H. Shih, C. T. Yeh and G. C. Yen, Effects of anthocyanidin on the inhibition of proliferation

- 468 and induction of apoptosis in human gastric adenocarcinoma cells, *Food and chemical*
469 *toxicology : an international journal published for the British Industrial Biological Research*
470 *Association*, 2005, **43**, 1557-1566.
- 471 11. J. S. Lee, Y. R. Kim, J. M. Park, Y. E. Kim, N. I. Baek and E. K. Hong, Cyanidin-3-glucoside
472 isolated from mulberry fruits protects pancreatic beta-cells against glucotoxicity-induced
473 apoptosis, *Molecular medicine reports*, 2015, **11**, 2723-2728.
- 474 12. X. Yan, L. Wu, B. Li, X. Meng, H. Dai, Y. Zheng and J. Fu, Cyanidin-3-O-glucoside Induces
475 Apoptosis and Inhibits Migration of Tumor Necrosis Factor- α -Treated Rat Aortic Smooth
476 Muscle Cells, *Cardiovascular Toxicology*, 2016, **16**, 251-259.
- 477 13. D. Liu, F. Pan, J. Liu, Y. Wang, T. Zhang, E. Wang and J. Liu, Individual and combined
478 antioxidant effects of ginsenoside F2 and cyanidin-3-O-glucoside in human embryonic kidney
479 293 cells, *RSC Adv.*, 2016, **6**, 81092-81100.
- 480 14. X. Yan, L. Wu, B. Li, X. Meng, H. Dai, Y. Zheng and J. Fu, Cyanidin-3-O-glucoside Induces
481 Apoptosis and Inhibits Migration of Tumor Necrosis Factor-alpha-Treated Rat Aortic Smooth
482 Muscle Cells, *Cardiovasc Toxicol*, 2015, DOI: 10.1007/s12012-015-9333-z.
- 483 15. N. Priego, M. Arechederra, C. Sequera, P. Bragado, A. Vázquezcarballo, Á. Gutiérrezzuzquiza, V.
484 Martínganado, J. J. Ventura, M. G. Kazanietz and C. Guerrero, C3G knock-down enhances
485 migration and invasion by increasing Rap1-mediated p38 α activation, while it impairs tumor
486 growth through p38 α -independent mechanisms, *Oncotarget*, 2016, **7**, 45060-45078.
- 487 16. A. D. Bangham, M. W. Hill and N. G. A. Miller, in *Methods in Membrane Biology: Volume 1*, ed.
488 E. D. Korn, Springer US, Boston, MA, 1974, DOI: 10.1007/978-1-4615-7422-4_1, pp. 1-68.
- 489 17. N. A. Lopes, C. M. B. Pinilla and A. Brandelli, Pectin and polygalacturonic acid-coated
490 liposomes as novel delivery system for nisin: Preparation, characterization and release
491 behavior, *Food Hydrocolloids*, 2017, **70**, 1-7.
- 492 18. R. Guan, J. Ma, Y. Wu, F. Lu, C. Xiao, H. Jiang and T. Kang, Development and characterization
493 of lactoferrin nanoliposome: cellular uptake and stability, *Nanoscale Research Letters*, 2012, **7**,
494 679.
- 495 19. M. Guan, Y. Zhou, Q.-L. Zhu, Y. Liu, Y.-Y. Bei, X.-N. Zhang and Q. Zhang, N-trimethyl chitosan
496 nanoparticle-encapsulated lactosyl-norcantharidin for liver cancer therapy with high targeting
497 efficacy, *Nanomedicine: Nanotechnology, Biology and Medicine*, 2012, **8**, 1172-1181.
- 498 20. L. Zou, S. Peng, W. Liu, X. Chen and C. Liu, A novel delivery system dextran sulfate coated
499 amphiphilic chitosan derivatives-based nanoliposome: Capacity to improve in vitro digestion
500 stability of (-)-epigallocatechin gallate, *Food Research International*, 2015, **69**, 114-120.
- 501 21. Y.-W. Noh, S.-Y. Kim, J.-E. Kim, S. Kim, J. Ryu, I. Kim, E. Lee, S. H. Um and Y. T. Lim, Multifaceted
502 Immunomodulatory Nanoliposomes: Reshaping Tumors into Vaccines for Enhanced Cancer
503 Immunotherapy, *Advanced Functional Materials*, 2017, **27**, 1605398.
- 504 22. D. W. Hutmacher, Biomaterials offer cancer research the third dimension, *Nat Mater*, 2010, **9**,
505 90-93.
- 506 23. H. Page, P. Flood and E. G. Reynaud, Three-dimensional tissue cultures: current trends and
507 beyond, *Cell Tissue Res*, 2013, **352**, 123-131.
- 508 24. N. Anastasov, I. Hofig, V. Radulovic, S. Strobel, M. Salomon, J. Lichtenberg, I. Rothenaigner, K.
509 Hadian, J. M. Kelm, C. Thirion and M. J. Atkinson, A 3D-microtissue-based phenotypic
510 screening of radiation resistant tumor cells with synchronized chemotherapeutic treatment,
511 *BMC Cancer*, 2015, **15**, 466.

- 512 25. J. G. Moffat, J. Rudolph and D. Bailey, Phenotypic screening in cancer drug discovery - past,
513 present and future, *Nat Rev Drug Discov*, 2014, **13**, 588-602.
- 514 26. J. A. Hickman, R. Graeser, R. de Hoogt, S. Vidic, C. Brito, M. Gutekunst, H. van der Kuip and I. P.
515 consortium, Three-dimensional models of cancer for pharmacology and cancer cell biology:
516 Capturing tumor complexity in vitro/ex vivo, *Biotechnology Journal*, 2014, **9**, 1115-1128.
- 517 27. J. M. Kelm, N. E. Timmins, C. J. Brown, M. Fussenegger and L. K. Nielsen, Method for
518 generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell
519 types, *Biotechnol Bioeng*, 2003, **83**, 173-180.
- 520 28. E. Cukierman, R. Pankov, D. R. Stevens and K. M. Yamada, Taking Cell-Matrix Adhesions to the
521 Third Dimension, *Science*, 2001, **294**, 1708.
- 522 29. M. Ravi, A. Ramesh and A. Pattabhi, Contributions of 3D Cell Cultures for Cancer Research, *J*
523 *Cell Physiol*, 2016, DOI: 10.1002/jcp.25664.
- 524 30. G. Y. Lee, P. A. Kenny, E. H. Lee and M. J. Bissell, Three-dimensional culture models of normal
525 and malignant breast epithelial cells, *Nat Methods*, 2007, **4**.
- 526 31. M. Vinci, S. Gowan, F. Boxall, L. Patterson, M. Zimmermann, W. Court, C. Lomas, M. Mendiola,
527 D. Hardisson and S. A. Eccles, Advances in establishment and analysis of three-dimensional
528 tumor spheroid-based functional assays for target validation and drug evaluation, *BMC*
529 *Biology*, 2012, **10**, 29.
- 530 32. M. Ravi, V. Paramesh, S. R. Kaviya, E. Anuradha and F. D. Solomon, 3D cell culture systems:
531 advantages and applications, *J Cell Physiol*, 2015, **230**, 16-26.
- 532 33. S. Breslin and L. O'Driscoll, The relevance of using 3D cell cultures, in addition to 2D
533 monolayer cultures, when evaluating breast cancer drug sensitivity and resistance,
534 *Oncotarget*, 2016, **7**, 45745-45756.
- 535 34. N. Falkenberg, I. Hofig, M. Rosemann, J. Szumielewski, S. Richter, K. Schorpp, K. Hadian, M.
536 Aubele, M. J. Atkinson and N. Anastasov, Three-dimensional microtissues essentially
537 contribute to preclinical validations of therapeutic targets in breast cancer, *Cancer Med*, 2016,
538 **5**, 703-710.
- 539 35. X. Luo, R. Guan, X. Chen, M. Tao, J. Ma and J. Zhao, Optimization on condition of
540 epigallocatechin-3-gallate (EGCG) nanoliposomes by response surface methodology and
541 cellular uptake studies in Caco-2 cells, *Nanoscale Res Lett*, 2014, **9**, 291.
- 542 36. T. Liang, R. Guan, H. Shen, Q. Xia and M. Liu, Optimization of Conditions for
543 Cyanidin-3-O-Glucoside (C3G) Nanoliposome Production by Response Surface Methodology
544 and Cellular Uptake Studies in Caco-2 Cells, *Molecules*, 2017, **22**, 457.
- 545 37. E. Arab Tehrani, C. J. Kahn, C. Baravian, B. Maherani, N. Belhaj, X. Wang and M. Linder,
546 Elaboration and characterization of nanoliposome made of soya; rapeseed and salmon
547 lecithins: application to cell culture, *Colloids and surfaces. B, Biointerfaces*, 2012, **95**, 75-81.
- 548 38. E. Roka, Z. Ujhelyi, M. Deli, A. Bocsik, E. Fenyvesi, L. Szenté, F. Fenyvesi, M. Vecsernyes, J.
549 Varadi, P. Feher, R. Gesztelyi, C. Felix, F. Perret and I. K. Bacskay, Evaluation of the Cytotoxicity
550 of alpha-Cyclodextrin Derivatives on the Caco-2 Cell Line and Human Erythrocytes, *Molecules*,
551 2015, **20**, 20269-20285.
- 552 39. F. Bonnier, M. E. Keating, T. P. Wrobel, K. Majzner, M. Baranska, A. Garcia-Munoz, A. Blanco
553 and H. J. Byrne, Cell viability assessment using the Alamar blue assay: a comparison of 2D and
554 3D cell culture models, *Toxicology in vitro : an international journal published in association*
555 *with BIBRA*, 2015, **29**, 124-131.

- 556 40. T. Kang, R. Guan, X. Chen, Y. Song, J. Han and J. Zhao, In vitro toxicity of different-sized ZnO
557 nanoparticles in Caco-2 cells, *Nanoscale Research Letters*, 2013, **8**, 496.
- 558 41. R. Guan, T. Kang, F. Lu, Z. Zhang, H. Shen and M. Liu, Cytotoxicity, oxidative stress, and
559 genotoxicity in human hepatocyte and embryonic kidney cells exposed to ZnO nanoparticles,
560 *Nanoscale Res Lett*, 2012, **7**, 602.
- 561 42. P. Shi, A. Laude and W. Y. Yeong, Investigation of cell viability and morphology in 3D
562 bio-printed alginate constructs with tunable stiffness, *J Biomed Mater Res A*, 2017, **105**,
563 1009-1018.
- 564 43. Z. Xiao, Y. Zhang, X. Chen, Y. Wang, W. Chen, Q. Xu, P. Li and F. Ma, Extraction, identification,
565 and antioxidant and anticancer tests of seven dihydrochalcones from Malus 'Red Splendor'
566 fruit, *Food Chem*, 2017, **231**, 324-331.
- 567 44. T. Kang, R. Guan, Y. Song, F. Lyu, X. Ye and H. Jiang, Cytotoxicity of zinc oxide nanoparticles
568 and silver nanoparticles in human epithelial colorectal adenocarcinoma cells, *LWT - Food*
569 *Science and Technology*, 2015, **60**, 1143-1148.
- 570 45. T. Liang, R. Guan, M. Tao, F. Lyu, G. Cao, M. Liu and J. Gao, In Vitro Toxicity of Zinc Oxide
571 Nanoparticles from Food Additives in Human Gastric Epithelium (GES-1) Cells, *Science of*
572 *Advanced Materials*, 2017, **9**, 1393-1400.
- 573 46. Y. Lin, H. Lin, S. Zhang, Y. Chen, M. Chen and Y. Lin, The role of active oxygen metabolism in
574 hydrogen peroxide-induced pericarp browning of harvested longan fruit, *Postharvest Biology*
575 *and Technology*, 2014, **96**, 42-48.
- 576 47. Z. Gao, C. Zhang, W. Tian, K. Liu, R. Hou, C. Yue, Y. Wu, D. Wang, J. Liu, Y. Hu and Y. Yang, The
577 antioxidative and hepatoprotective effects comparison of Chinese angelica
578 polysaccharide(CAP)and selenizing CAP (sCAP) in CCl₄ induced hepatic injury mice,
579 *International Journal of Biological Macromolecules*, 2017, **97**, 46-54.
- 580 48. H. Chen, H. Hu, D. Chen, J. Tang, B. Yu, J. Luo, J. He, Y. Luo, J. Yu and X. Mao, Dietary Pectic
581 Oligosaccharide Administration Improves Growth Performance and Immunity in Weaned Pigs
582 Infected by Rotavirus, *J Agric Food Chem*, 2017, DOI: 10.1021/acs.jafc.7b00039.
- 583 49. J. Sudimack and R. J. Lee, Targeted drug delivery via the folate receptor, *Advanced Drug*
584 *Delivery Reviews*, 2000, **41**, 147-162.
- 585 50. I. Mahapatra, J. Clark, P. J. Dobson, R. Owen and J. R. Lead, Potential environmental
586 implications of nano-enabled medical applications: critical review, *Environmental Science:*
587 *Processes & Impacts*, 2013, **15**, 123-144.
- 588 51. T. Liang, R. Guan, Z. Wang, H. Shen, Q. Xia and M. Liu, Comparison of anticancer activity and
589 antioxidant activity between cyanidin-3-O-glucoside liposomes and cyanidin-3-O-glucoside in
590 Caco-2 cells in vitro, *RSC Advances*, 2017, **7**, 37359-37368.
- 591 52. P. Strugała, A. Dudra and J. Gabrielska, Interaction between Mimic Lipid Membranes and
592 Acylated and Nonacylated Cyanidin and Its Bioactivity, *Journal of Agricultural & Food*
593 *Chemistry*, 2016, **64**, 7414-7422.

595 Figure legends

596 Fig. 1. Transmission Election Microscope (TEM) image of C3G nanoliposomes.

597 Fig. 2. Cell morphology of 3D culture cells in different magnification times.

598 Fig.3. The cell morphology of 2D and 3D cell culture models(A:The cells culture in
599 2D cell culture model;B:The cells culture in 2D cell culture model that treated with
600 pancreatin ;C: The cells culture in 3D cell culture model.)

601 Fig.4. The cell viability of 2D and 3D culture cells expose to C3G and C3G liposomes
602 (A:The cell treated with C3G;B:The cell treated with C3G liposomes.)

603 Fig.5. The concentration of GSH in different cell culture models

604 Fig. 6. The activity of SOD in different cell culture models

605 Fig. 7. The content of MDA in different cell culture models

606 Fig. 8. The strength of antioxidant capacity in different cell culture models

Figure.1

Figure.2

Figure.3

620

621 (* $p < 0.05$, compared with the control group , # $p < 0.05$, compared with the 2D group, ^a $p < 0.05$,
 622 compared with the different dose group)

623

Figure.4

624

625 (^{*} $p < 0.05$,compared with the control group ,[#] $p < 0.05$,compared with the 2D group, ^a $p < 0.05$,

626 compared with the H₂O₂ treated group, ^b $p < 0.05$,compared with the 2D group)

627

Figure.5

628

629

630 (^{*} $p < 0.05$,compared with the control group ,[#] $p < 0.05$,compared with the 2D group, ^a $p < 0.05$,

631 compared with the H₂O₂ treated group, ^b $p < 0.05$,compared with the 2D group)

632

Figure.6

633

634 (* $p < 0.05$,compared with the control group ,[#] $p < 0.05$,compared with the 2D group, ^a $p < 0.05$,

635 compared with the H₂O₂ treated group)

636

Figure.7

637

638 (* $p < 0.05$,compared with the control group ,[#] $p < 0.05$,compared with the 2D group, ^a $p < 0.05$,

639 compared with the H₂O₂ treated group, ^b $p < 0.05$,compared with the 2D group)

640

Figure.8