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1 **Microbial sophorolipids inhibit colorectal tumour cell growth *in vitro* and restore haematocrit in Apc^{min+/-}**
2 **mice**

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26

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31 **Abstract**

32

33 Sophorolipids are glycolipid biosurfactants consisting of a carbohydrate sophorose head with a fatty acid tail and
34 exist in either an acidic or lactonic form. Sophorolipids are gaining interest as potential cancer chemotherapeutics
35 due to their inhibitory effects on a range of tumour cell lines. Currently, most anti-cancer studies reporting the
36 effects of sophorolipids have focused on lactonic preparations with the effects of acidic sophorolipids yet to be
37 elucidated. We produced a 94% pure acidic sophorolipid preparation which proved to be non-toxic to normal
38 human colonic and lung cells. In contrast we observed a dose-dependent reduction in viability of colorectal cancer
39 lines treated with the same preparation. Acidic sophorolipids induced apoptosis and necrosis, reduced migration
40 and inhibited colony formation in all cancer cell lines tested. Furthermore, oral administration of 50 mg kg⁻¹ acidic
41 sophorolipids over 70 days to Apc^{min/+} mice was well tolerated and resulted in an increased haematocrit, as well
42 as reducing splenic size and red pulp area. Oral feeding did not affect tumour numbers or sizes in this model. This
43 is the first study to show that acidic sophorolipids dose-dependently and specifically reduces colon cancer cell
44 viability in addition to reducing tumour-associated bleeding in the Apc^{min/+} mouse model.

45

46 **Keywords:** Sophorolipids, Acidic, Colorectal, Cancer, Apc^{min/+}

47

48 **Statements and Declarations**

49

50 Competing interests: The authors declare that they have no competing interests.

51

52 Ethical approval: The article does not contain any studies with human participants where ethical was required.

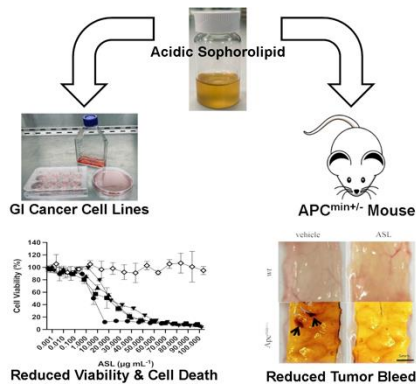
53 Experiments using animals were carried out after obtaining ethical approval and under licence in accordance with
54 the Animals (Scientific Procedures) Act 1986, U.K. Further detail is provided in the material and methods section.

55

56 Data availability statement: The datasets generated during and/or analysed during the current study are available
57 from the corresponding authors on reasonable request.

58

59 **Graphical Abstract**



60

61

62 **Key Points**

63

64 Acidic sophorolipids are produced by yeast species such as *Starmerella bombicola*.

65 Acidic sophorolipids selectively killed colorectal cells with no effect on healthy gut epithelia.

66 Acidic sophorolipids reduced tumour associated gut bleed in a colorectal mouse model.

67

68 **Introduction**

69

70 Familial Adenomatous Polyposis (FAP) is an autosomal dominant hereditary form of Colorectal cancer was
 71 responsible for over 10% of global cancer incidence and 9.4% of global cancer related deaths in 2020 (Ferlay et
 72 al. 2020). FAP is characterised by the development of numerous adenomas along the colorectal tract. Currently,
 73 the gold standard for treatment of FAP is surgery, followed by adjuvant chemotherapy (O’Connell et al. 2010).
 74 Adjuvant chemotherapy does not however discriminate between normal and transformed tissue, which leads to a
 75 variety of potentially serious complications including cardiotoxicity, immune dilapidation, and neurotoxicity
 76 (Morgan and Rubin 1998; Mazevet et al. 2013). To allay this problem, compounds that are non-toxic, orally
 77 tolerated and specifically target epithelial neoplastic cells in the intestinal tract could have great potential in
 78 delaying progression of intestinal neoplasms; in particular, those that are associated with progression to these
 79 colorectal cancers (Kelloff et al. 1994; D’Incalci et al. 2005).

80 There is an increasing body of research demonstrating compounds derived from natural sources such as
 81 plants and microbial secondary metabolites have preventative or targeted anti-cancer activity (Fridlender et al.
 82 2015; Khalifa et al. 2019). An example that is already in widespread in cancer treatment is Paclitaxel, first derived
 83 from the bark of the yew tree (*Taxus baccata*) (Stierle et al. 1993) and is now used for the treatment of several
 84 different cancer types including ovarian, oesophageal, breast, lung and pancreatic cancer (Barbuti and Chen 2015).

85 Sophorolipids are a class of naturally occurring glycolipids produced by yeast species such as *Starmerella*
86 *bombicola* (Van Bogaert et al. 2007; Roelants et al. 2019). Comprising a hydrophilic sophorose moiety covalently
87 linked to hydrophobic hydroxylated fatty acid tails ranging between 16 and 18 carbons in length, sophorolipid
88 congeners are produced in two main forms either lactonic (LSL) or acidic (ASL) (Fig. S1) (Banat et al. 2010;
89 Marchant and Banat 2012a; Marchant and Banat 2012b). During fermentation yeast species generate mixtures of
90 these structurally different congeners (Van Bogaert *et al.*, 2007). The anti-cancer properties of sophorolipids have
91 received a lot of attention in recent years. Sophorolipids and their synthetically synthesised derivatives have shown
92 *in vitro* cytotoxic effects in human pancreatic (HPAC), liver (H7402), lung (A549), brain (LN229, HNCG-2),
93 oesophageal (KYSE109, KYSE450), breast, cervical (HeLa), leukaemic (HL60, K562) and melanoma (SK-
94 MEL-28) cell lines (Chen et al. 2006a; Chen et al. 2006b; Fu et al. 2008; Dhar et al. 2011; Shao et al. 2012;
95 Ribeiro et al. 2015; Li et al. 2017; Ceresa et al. 2021; Adu et al. 2022). Sophorolipids have also shown potential
96 tumour shrinking capability in an *in vivo* model of cervical cancer (Li et al. 2017). However, to date, very few *in*
97 *vivo* bioactivity studies have been reported for sophorolipid mixtures. A small number of toxicology experiments
98 have shown that sophorolipids are non-irritating when topically applied to the skin and eyes of rabbits and non-
99 toxic when administered orally to either mice or rats (Ikeda et al. 1986; Callaghan et al. 2016). Sophorolipid
100 mixtures have also been observed to reduce inflammation and reduce mortality rates in a rat model of severe
101 abdominal sepsis as well as decreasing IgE levels in a murine asthma model (Hardin et al. 2007; Bluth et al. 2008).

102 Although sophorolipids show promising anti-cancer activity *in vitro*, the gross composition and
103 percentage of congeners within the sophorolipid mixture used in most investigations are not fully disclosed. It can
104 be assumed that LSL enriched preparations are more common, due to the preponderance of LSL's in comparison
105 to ASL's within naturally occurring sophorolipid mixtures (Van Bogaert et al. 2011; Marchant and Banat 2012a).
106 The composition of sophorolipid preparations is an important factor to consider when investigating the targeted
107 or preventative anti-cancer effect of these molecules. The reasoning for this is twofold; firstly, to reduce toxicity
108 resulting from contaminants in crude preparations and, natural products being investigated must be composed
109 completely of highly purified biologically active agent (Beutler 2019; Adu et al. 2022). In the case of paclitaxel
110 and other naturally derived agents, this has necessitated the synthetic production in the laboratory of the active
111 agent with targeted anti-cancer properties (Flam 1994). Secondly, and arguably more important, there is
112 significant difference in the functional properties attributed to LSL and ASL congeners. LSL's have strong
113 antimicrobial action that is not seen in ASL's (Van Bogaert et al. 2007; Elshikh et al. 2017). Previously, we have
114 shown that a highly purified preparation of LSL results in exacerbation of adenomatous tumour growth in the

115 intestinal tract of a colorectal cancer murine model, ($Apc^{min/+}$ mouse), with secondary consequences including
116 splenomegaly and reduced haematocrit levels (Callaghan et al. 2016).

117 To date, the anti-cancer bioactivity of purified ASL's have only been investigated within melanoma cell
118 lines (Adu et al. 2022). In contrast to our previous work with LSL, an ASL specific targeted anti-cancer effect
119 would be advantageous for several reasons. In comparison to LSL congeners, ASL's have reduced production
120 costs as they are the first congeners to be produced in the bioreactor when less favourable conditions that require
121 a reduced energy requirement are present (Casas and García-Ochoa 1999; Sarubbo et al. 2022). The proportion of
122 ASL within enriched preparations can be improved when the medium is supplemented with polyunsaturated fatty
123 acids and this can be further increased via simple hydrolysis of the sophorolipid mixtures which increases the
124 number of open ring structures (Van Bogaert et al. 2011). Alternatively, a modified strain lacking the lactonising
125 enzyme can be applied. Yet, in contrast to the hydrolysis strategy, acetylation of the glucose moieties is still
126 possible (Roelants *et al.*, 2016). ASL's have enhanced solubility when compared to other sophorolipid congeners,
127 which may prove useful for their use as pharmaceuticals, as they can be dissolved in saline instead of potentially
128 toxic solvents such as DMSO (Baccile et al. 2016). Both the solubility as well as enhanced foaming attributes of
129 ASL's contribute to their preferred use in the food, cosmetic and the bioremediation industries (Roelants et al.
130 2016; Naughton et al. 2019; Adu et al. 2020).

131 In this study we investigated the targeted therapeutic effect of purified ASL *in vitro* in human colorectal
132 cancer cell lines and their ability to inhibit tumour growth in $Apc^{min/+}$ mice. The $Apc^{min/+}$ mouse model of FAP
133 was used as it recapitulates key pathological features of the human disease and provides a useful tool to investigate
134 the effects of genetics, diet, and therapeutic drugs on tumorigenesis in the gastrointestinal tract (Hinoi et al. 2007).
135 Like human FAP, it was first noted that $Apc^{min/+}$ mice develop adult-onset anaemia with haematocrit levels <45%,
136 the passage of bloody stools, and have a reduced life span (Moser et al. 1995). Additionally, post-mortem analysis
137 revealed the growth of numerous tumours along the small intestine (Moser et al. 1995). The $Apc^{min/+}$ mouse model
138 has also been widely used to test the effects of chemotherapeutics on tumour growth and development such as
139 NSAIDs (Aspirin) (Reuter et al. 2002). In addition, the $Apc^{min/+}$ mouse model has also been used to develop a
140 further understanding of clinically used chemotherapeutic drugs such as 5-fluorouracil, which is widely used in
141 the treatment of CRC (Tucker et al. 2002). Therefore, we hypothesize that a highly purified and well characterised
142 preparation of ASL (94% pure diacetylated) will selectively inhibit colorectal tumour growth *in vitro* and delay
143 disease progression in the $Apc^{min/+}$ mouse model.

144

145 **Material and Methods**

146

147 ASL production and purification.

148

149 ASL's used in this study were generated in house from a crude precursor sophorolipid mixture, (*Sopholiance S*,
150 Batch N°11103A), purchased from *Givaudan* (Vernier, Switzerland). ASL congeners were obtained according to
151 a method described by Baccile *et al.* (2013). In brief, sophorolipids were purified from *Sopholiance S* via liquid
152 phase extraction in acidified ethyl acetate followed by hexane washing. Following purification, the sophorolipid
153 material was subjected to alkaline hydrolysis by diluting in 5 M NaOH (*Merck*) and heating under reflux to 90°C
154 for 10 min. The material was then acidified to pH 4 using 18.5% (w/v) HCl (*Merck*). ASL was then recovered via
155 precipitation in a pentanol / hexane medium (*Merck*) at -18°C (Baccile *et al.* 2013). Following production and
156 purification, SL congeners were identified by a UHPLC system with RS Diode Array detector (*ThermoFisher*
157 *Scientific*) in conjunction with the amaZon SL dual funnel Ion Trap spectrometer LCMS system (*Bruker*). The
158 percentage relative amounts of each congener were calculated and a ratio of ASL to LSL determined (Smyth *et*
159 *al.*, 2010).

160

161 Cell culture

162

163 Colorectal cancer cell lines HT29 (ATCC® HTB-38), HT115 (ECACC 85061104), HCT116 (ATCC® CCL-247),
164 and Caco2 (ATCC® HTB-37) as well as colonic epithelium CCD-841-CoN (ATCC® CRL-1790) cell lines were
165 used in this study. Cell lines were maintained in DMEM low glucose media or MEM low glucose media
166 (*ThermoFisher Scientific*) supplemented with 10% (v/v) foetal bovine serum (*ThermoFisher Scientific*). All
167 cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

168

169 Cell viability assay

170

171 A total of 1 x 10⁴ cells per well were seeded in to a 96 well plate (*Sarstedt*) and allowed to attach overnight before
172 being serum starved for 24 hours. Cells were then treated with concentrations of ASL ranging between 0.001 µg
173 mL⁻¹ – 100 µg mL⁻¹ or vehicle-only control (PBS) for 24 hours. Subsequently, 10 µL of a 25 mg mL⁻¹ solution of
174 MTT (3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) (*Merck*) was added to each well and the

175 plate further incubated for 1 hour at 37°C. Formazan crystals were solubilized with 100 μ L of DMSO (*Merck*).
176 Absorbance at 570 nm was measured using a FLUOstar Omega microplate reader (*BMG-LABTECH*).
177 Experiments were repeated three times with six internal replicates per treatment group. Data are representative of
178 the three independent repeats and presented as mean percentage reduction in absorbance in comparison to vehicle-
179 only controls \pm standard deviation (SD).

180

181 Visualisation of morphological changes induced by ASL treatment

182

183 A total of 1×10^4 cells per well were seeded on to a 96 well plate (*Sarstedt*) and allowed to attach overnight
184 forming a monolayer before being serum starved for 24 hours. Cells were then treated with both 20 and 70 μ g mL⁻¹
185 of ASL or vehicle-only control (PBS) for 24 hours. The cells were subsequently imaged with an Axio Scope 1
186 microscope (*Zeiss*) at 200 \times magnification. Images obtained from three random fields were selected and assessed
187 for morphological changes by comparing each cell line to the vehicle-only control, looking for changes in cell
188 shape and confluency. Experiments were plated in triplicate and repeated three times.

189

190 Quantification of detached cells

191

192 A total of 1×10^4 cells per well were seeded on to a 96 well plate (*Sarstedt*) and allowed to attach overnight
193 forming a monolayer before being serum starved for 24 hours. Cells were then treated with both 40 and 70 μ g mL⁻¹
194 of ASL or vehicle-only control (PBS) for 24 hours. 10 μ g of supernatant was removed from each well, placed
195 into a 1.5 mL microtube (*Sarstedt*). Cells were stained with 5 μ M of both Syto 9 and propidium iodide (*Merck*)
196 by adding directly into the microtube and incubating at room temperature for 30 minutes. The cells were then
197 centrifuged at 150 $\times g$ for 5 minutes and the supernatant was aspirated. The remaining cells pellet was washed
198 using ice-cold PBS (pH 7.4) (*ThermoFisher Scientific*) and spun onto a microscope slide using a Shandon
199 cytocentrifuge (*ThermoFisher Scientific*) for five minutes at 176 $\times g$. Slides were subsequently imaged with a
200 Axio Scope 1 microscope (*Zeiss*) at 400 \times magnification. Following staining with Syto9 and propidium iodide,
201 fluorescent staining was present in nucleus therefore live cells appeared green while dead cells fluoresced red
202 (Altman et al. 1993) and as such cells were counted. A minimum of 1 cell and a maximum of 250 cells were
203 counted per field of view. Experiments were plated in triplicate and repeated three times, data set shown as mean
204 \pm Standard Error of the Mean (SEM).

205

206 Acridine orange / ethidium bromide staining and quantification

207

208 To determine the mechanism of cell death induced by addition of ASL, cells were stained *in situ* with acridine
209 orange at 10 mg mL⁻¹ (*Merck*) and ethidium bromide 1 mg mL⁻¹ (*Merck*). Morphological changes were assessed
210 by fluorescence microscopy. For assessment of apoptosis, a total of 3 × 10⁴ cells were seeded onto a 10 mm
211 coverslip (*Agar Scientific*) placed within a 6 well plate and the cells were incubated overnight to form a confluent
212 monolayer. Following serum starvation for 24 hours; ASL (at a concentration of either 20 µg mL⁻¹ or 70 µg mL⁻¹)
213 ¹), vehicle control (PBS), or 5 µM of etoposide (apoptotic control) (*Merck*) was added and the plate incubated for
214 a further 24 hours. To determine the number of live cells remaining on the coverslip, the samples were washed
215 three times with ice-cold phosphate buffered saline at pH7.4 (*ThermoFisher Scientific*), followed by incubation
216 with 10 µL of a solution of acridine orange / ethidium bromide with a volumetric ratio of 1:1 for 5 minutes.
217 Finally, the cells were washed again three times with ice-cold PBS at pH7.4 (*ThermoFisher Scientific*). Cells were
218 subsequently imaged with a Axio Scope 1 fluorescence microscope (*Zeiss*) at 400 × magnification. A total of 300
219 attached cells/coverslip were morphologically identified and counted as necrotic (red/orange nuclei), apoptotic
220 (green condensed or fragmented nuclei) or live (green non-condensed ovoid or rounded nuclei). The operator was
221 blinded to the experimental groups and random fields were selected. Each experiment was replicated three times
222 with six internal repeats per group. Data are representative of three independent repeats and presented as mean ±
223 SEM.

224

225 Scratch assay

226

227 For wound healing scratch assays, 1.6 × 10⁶ cells were plated in each well of a 6 well plate (*Sarstedt*) and allowed
228 to attach overnight. Cells were serum starved for 24 hours and treated with 5 µg mL⁻¹ of mitomycin C (*Merck*)
229 for 2hours prior to the scratch to inhibit proliferation. The “wound” was made by scratching a line in the centre
230 of the confluent monolayer using a sterile toothpick. Cells were rinsed very gently three times with PBS at pH7.4
231 (*ThermoFisher Scientific*) and cultivated in serum free media supplemented with PBS vehicle-control or 10 - 20
232 µg mL⁻¹ ASL for up to 72 hours. The cell was imaged at various time points using a ELWD TI SCP microscope
233 (*Nikon Europe B. V.*) at 100 × magnification. To quantify migration of cells into the scratch wound, the area of
234 the gap was measured using ImageJ software (Schneider et al. 2012). After 72 hours, the area of the remaining

235 gap was measured and the difference between initial and final areas calculated. Experiments were repeated three
236 times using triplicate technical replicates per experimental group. Data are representative of the three independent
237 repeats and presented as mean \pm SEM.

238

239 Chemotactic cell migration assays

240

241 Chemotactic cell migration assays were carried out in a modified Boyden chamber, based on a previously
242 described protocol (Chen 2005). 1×10^4 cells per well of CCD-841-CoN, HT29 or HT115 CRC cells were placed
243 in the upper compartment of a 96- well FluoroBlok transwell inserts (*Analab*) which contained 8 μ m pore size
244 polyethylene terephthalate filters (*Analab*) and the cells were allowed to attach overnight. A final concentration
245 of 0, 10 or 50 μ g mL⁻¹ of ASL in serum free media was added to the upper chamber, while appropriate culture
246 media supplemented with 10% FBS was added (as a chemoattractant) to the bottom chamber. Cells were left to
247 migrate overnight in an incubator at 37°C and at 5% CO₂. Following incubation, all media in the upper chamber
248 was removed and all cells that failed to migrate were removed using a sterile cotton swab. Cells that had migrated
249 through to the lower surface of the filter insert were stained with a 0.1% (w/v) solution of crystal violet (*Merck*)
250 made up in 25% (v/v) methanol (*Merck*). A total of three random areas were chosen and a minimum of 1 cell and
251 a maximum of 300 cells stained with crystal violet were counted under a Axio Scope 1 light microscope (*Zeiss*)
252 at 400 \times magnification. Migration rates were expressed as total percentage of the control. Data are representative
253 of the three independent repeats and presented as mean \pm SEM.

254

255 Animal model

256

257 Prior to the commencement of the study all animal procedures were approved by both the animal care and ethics
258 committee at Ulster University and the UK Home Office. Additionally, all animal procedures were carried out by
259 licensed personnel in accordance with both local animal welfare committee (Ulster University) and national (UK
260 Home Office) guidelines (Workman et al. 2010). For breeding purposes male *Apc*^{min+/-} mice were housed together
261 with female wild type (*wt*) mice and all the animals subjected to a 12/12 light cycle, with food and water being
262 available *ad libitum*. Mice were genotyped as described previously in Callaghan *et al.*, (2016), with both
263 heterozygous *Apc*^{min+/-} and *wt* mice (male and female) used in experiments. During the study mice were monitored
264 daily for grooming activity, general behaviour, activity levels, food and water intake and general wellbeing. Mice

265 assessed as exhibiting signs of distress or discomfort were immediately removed from the study and euthanized
266 via CO₂ asphyxiation.

267

268 ASL dosing

269

270 At five weeks of age, both *wt* littermate and *Apc*^{min/+} mice were treated via oral gavage every other day with either
271 vehicle-only or a solution containing 50 mg kg⁻¹ (body weight) of ASL suspended in saline for 70 days. During
272 treatment, body weights, general health and behaviour were monitored bi-weekly. Food and water were weighed
273 on a weekly basis to determine the effect of ASL treatment on eating/drinking habits. A cut-off point for body
274 weight reduction of 10% was applied and mice reaching this point were euthanized via CO₂ euthanasia.

275

276 Tissue collection and assessment

277

278 At the end of the experimental protocol, mice were euthanized with an overdose of pentobarbitone (200 mg kg⁻¹
279 given IP). A cardiac puncture was performed to collect blood that was stored in EDTA tubes (*Aquilant Scientific*).
280 An additional blood sample was taken for haematocrit level estimation. For this a blood capillary tube was filled
281 with blood, sealed, and centrifuged (Microcentrifuge with Hematocrit Rotor; *Cole-Parmer*) at 13,000 × *g* for 5
282 minutes. Haematocrit levels were calculated using the following calculation: height of RBC/total height of all the
283 components × 100. Internal organs including the intestinal tract, colon, spleen, heart, liver, kidneys and lungs
284 were removed, weighed and immerse fixed in 10% buffered formal saline at pH7.4 (*ThermoFisher Scientific*).
285 The intestinal tracts were divided into 3 sections according to the description provided by Casteleyn *et al.*, (2010).
286 After identification of the specific intestinal regions, samples were bisected longitudinally, and the total number
287 of polyps was recorded as well as their diameters measured with callipers (Casteleyn *et al.* 2010). The specimens
288 were then cut into ~2 cm strips and placed in cassettes prior to standard wax embedding. To assess qualitative
289 histopathological changes in the intestines and spleen, tissues were cut into 5 μm sections using a Shandon Finesse
290 325 Microtome (*ThermoFisher Scientific*) placed on glass slides, cleared with xylene, dehydrated in descending
291 grades of ethanol and subsequently stained with Mayer's haematoxylin and eosin (*Merck*). Stained sections were
292 examined with a Axio Scope 1 light microscope (*Zeiss*) at a range of objective magnifications.

293

294 Statistical analyses

295

296 Statistical analysis of cell viability data was determined by two-way ANOVA followed by both Tukey's and
297 Dunnett's post hoc testing. Analysis of live/ dead cell counts was determined by two-way ANOVA followed by
298 Šidák's post hoc testing. Analysis of remaining *in vitro* data was determined by two-way ANOVA followed by
299 both Dunnett's post hoc testing. Comparisons between *in vivo* groups was assessed using a students' t-test. A
300 value of $p \leq 0.05$ was considered statistically significant. All statistical analysis was carried out with the aid of
301 Prism Version 9.3.1 (350) (*GraphPad Software*).

302

303 **Results**

304

305 Production and purification of ASL

306

307 A purified form of diacetylated ASL was produced for use in in this study from a commercially available SL
308 formulation using methodologies outlined by Baccile *et al.* (2013). Following the recommendation of Twigg *et*
309 *al.* 2021, the purified sample was analyzed via HPLC-ELSD and was found to be composed of 94% nonacetylated
310 ASL. A breakdown of the various nonacetylated ASL congeners present in the samples can be seen in Table S1.
311 The most abundant congener present in the sample was acidic C18:1s (75% relative abundance). The acidic C18:1t
312 and C18:2t congeners represented 10% each, while all other ASL congeners were limited to less than 2% relative
313 abundance (Twigg *et al.* 2021).

314

315 ASL have a selective effect on colorectal cancer cell viability.

316

317 Several colorectal tumour cell lines (HT29, HT115, HCT116 and Caco2), in addition to a colonic epithelial cell
318 line (CCD-841-CoN) were treated with ASL to assess the effect on cell viability. ASL at concentrations up to 100
319 $\mu\text{g mL}^{-1}$ effected no significant decrease in the viability of the CCD-841-CoN cell line after 24 hours of treatment
320 in comparison to cultures treated with a vehicle-only control (PBS) (Fig. 1a). However, 24 hours of treatment with
321 ASL concentrations equal to or above 1 $\mu\text{g mL}^{-1}$ resulted in significantly reduced viability in the HT115 cell line
322 in comparison to vehicle-only controls ($p = 0.0201$) (Fig. 1a). In HT29, Caco2 and HCT116 cell lines, treatment
323 with ASL concentrations equal to or above 10 $\mu\text{g mL}^{-1}$ resulted in a significant reduction in cell viability in
324 comparison with cells treated with vehicle-only controls ($p = < 0.0001$, < 0.0001 , and 0.0099 respectively) (Fig.

325 1a). The viability of colorectal tumour cell lines HT29, HT115 and Caco2 were significantly reduced when
326 compared to the colonic epithelial cell line CCD-841-CoN when treated with ASL concentrations equal to or
327 above $1 \mu\text{g mL}^{-1}$ ($p = 0.0146, < 0.0001, \text{ and } 0.0017$ respectively). For cell line HCT116 a significant reduction in
328 viability when compared to CCD-841-CoN was observed when cells were treated with ASL concentrations equal
329 to or above $10 \mu\text{g mL}^{-1}$ ($p = 0.0046$) (Fig. 1a).

330

331 ASL induces cell rounding, detachment and cell death in colorectal cancer cells

332

333 To observe potential effects on cell morphology, colorectal cancer cell lines (HT29, HT115, HCT116 and Caco2)
334 and the colonic epithelial cell line (CCD-841-CoN) were treated with ASL and assessed by microscopic
335 examination. CCD-841-CoN cells treated with a vehicle-only control (PBS) grew as a confluent monolayer with
336 a bipolar morphology (Fig. S2). Following treatment with either 20 or $70 \mu\text{g mL}^{-1}$ ASL no morphological
337 differences of cells in the monolayer were observed when compared to vehicle-only treated CCD-841-CoN cells
338 (Fig. S2). HT29, HT115, HCT116 and Caco2 treated with the vehicle-only control displayed a densely packed,
339 cobblestone-like monolayer morphology (Fig. S2). When treated with ASL, disruption to the monolayer and
340 detachment of HT-29, HT115, HCT116 and Caco-2 cell lines was observed. Partially detached and rounded cells
341 were conspicuous within HT29, HT115 and Caco2 cell lines treated with $20 \mu\text{g mL}^{-1}$ (Fig. S2). HT29, HT115,
342 HCT116 and Caco2 cells exposed to $70 \mu\text{g mL}^{-1}$ ASL, demonstrated extensive monolayer disruption with
343 distinctive cell free areas within the cultures (Fig. S2).

344 As cellular detachment was observed in all tumour cell cultures, cells were isolated from the supernatant
345 and stained with syto9 and propidium iodine to determine if detachment resulted in cell death. Only a small
346 number of CD-841-CoN cells treated with either concentration of ASL were observed to detach and there was no
347 significant difference in the proportion of these detached cells that were either alive or dead (Fig. 1b). In contrast
348 the number of detached HT29, HT115, HCT116 and Caco2 cells was higher when treated with either
349 concentration of ASL when compared to vehicle-only treated cells, and there was a significant increase in the
350 proportion of these detached cells that were found to be dead (Fig. 1b).

351

352 ASL induces cell death *in vitro* via both apoptotic and necrotic pathways.

353

354 As treatment with ASL resulted in a significant loss of cell viability, and induced detachment and cell death in
355 colorectal cancer cell lines, the mechanism of cell death was investigated. Colorectal cancer (HT29, HT115 and
356 HCT116 and Caco2) and colonic epithelial (CCD-841-CoN) cells lines were treated with ASL and stained with
357 acridine orange and ethidium bromide to determine whether cell death was morphologically identifiable as a result
358 of apoptosis or necrosis. Treatment of CCD-841-CoN cells with 20 $\mu\text{g mL}^{-1}$ of ASL did not result in a significant
359 increase in the numbers of either apoptotic or necrotic cells. However, treatment with 70 $\mu\text{g mL}^{-1}$ ASL resulted
360 in 10% cell death within the culture that was identified morphologically as apoptosis ($p < 0.05$) (Fig. 1c). A dose-
361 dependent increase in cell death in all four human colorectal cancer cell lines was observed following ASL
362 treatment (Fig. 1C). In HT29, HT115 and HCT116 cell lines treated with 20 $\mu\text{g mL}^{-1}$ ASL, there were equivalent
363 numbers of apoptotic and necrotic cells. However, there was a higher number of apoptotic cells in Caco2 cells
364 treated with 20 $\mu\text{g mL}^{-1}$ ASL ($p < 0.01$) (Fig. 1c). Exposure of cultures to 70 $\mu\text{g mL}^{-1}$ ASL, resulted in a markedly
365 reduced number of adherent colorectal cancer cells available for quantification. HT29 and HT115 cultures treated
366 with 70 $\mu\text{g mL}^{-1}$ showed a significant increase in the numbers of apoptotic and necrotic cells ($p < 0.001$, $p < 0.01$
367 respectively) while HCT116 cells were predominantly necrotic ($p < 0.0001$), and Caco-2 cells had a higher number
368 of apoptotic cells ($p < 0.001$) compared to vehicle-only controls (Fig. 1c).

369

370 ASL reduces motility and anchorage-independent growth of tumour cells.

371

372 To assess the effect of ASL on cell migration, a scratch was made across a monolayer of colorectal cell lines HT29
373 and HCT115, and the colonic epithelial cell line CCD-841-CoN. Treatment with 10 $\mu\text{g mL}^{-1}$ ASL had no
374 significant effect on CCD-841-CoN cell migration, with 90% of the total scratch area being covered after 72 hours
375 (Fig. 2a). In contrast, treatment with 10 $\mu\text{g mL}^{-1}$ ASL resulted in a highly significant decrease in the proportion
376 of the total scratch area covered in HT-29 (10%; $p < 0.0001$) and HT115 cells (22%; $p < 0.0001$) after 72 hours
377 (Fig. 2a). To test the effect ASL had on chemotaxis, the same cell lines were plated in the upper portion of a
378 Boyden chamber and the number of cells migrating in response to FCS were counted after 24 hours. Media
379 supplemented with either 10 or 50 $\mu\text{g mL}^{-1}$ ASL did not significantly affect the migration of CCD-841-CoN cells.
380 The addition of 10 $\mu\text{g mL}^{-1}$ ASL to media did however result in a significant reduction in migration of HT29
381 (33%) and HT115 (29%) compared to control values ($p < 0.001$) (Fig. 2b). The addition of 50 $\mu\text{g mL}^{-1}$ ASL
382 reduced migration of HT29 and HT115 to 8.3% and 10.4% of control values respectively ($p < 0.0001$) (Fig. 2b).

383

384 *In vivo* experiments: *wt* and *Apc*^{min+/-} mice tolerate the oral administration of ASL

385

386 To determine palatability and potential toxicity of ASL, a pilot study was performed with both *wt* and *Apc*^{min+/-}
387 mice which were fed either a vehicle-only control solution consisting of 10% sucrose water or vehicle containing
388 either 0.5, 5 or 50 mg kg⁻¹ ASL for a period of 5 weeks (n = 3/group). Both *wt* and *Apc*^{min+/-} mice gained weight
389 at a similar rate and there was no change in food or water intake between any of the treatment groups (Fig. S3)
390 On completion of this oral feeding tolerance study, the major organs (liver, stomach, kidneys, lungs, heart, spleen
391 and pancreas) were removed, weighed and a gross inspection of morphology was carried out. There were no
392 significant differences in organ weights (Table S2) or gross organ morphology observed between mice fed on the
393 vehicle only control diet or on ASL.

394

395 ASL reduces polyp-associated bleeding and increases haematocrit levels but does not affect tumour size or
396 numbers in *Apc*^{min+/-} mice.

397

398 The gross morphological appearances of unfixed, flat-mounted ilea from *wt* mice treated with either vehicle-only
399 control or 50 mg kg⁻¹ ASL was characterised by a flattened, uniformly smooth mucous epithelium with prominent
400 patent blood vessels (Fig. 3a). In vehicle-only treated *Apc*^{min+/-} mice, there was clear evidence of polyp-associated
401 bleeding within the ileal segment (Fig. 3a). In contrast, *Apc*^{min+/-} mice treated with 50 mg kg⁻¹ ASL for 70 days,
402 showed little evidence of bleeding from these intestinal polyps (Fig. 3a). However, the number of intestinal polyps
403 in *Apc*^{min+/-} mice was not significantly different following treatment with 50 mg kg⁻¹ ASL for 70 days in
404 comparison the vehicle-only control treated cohort (vehicle = 48 ± 2 vs ASL = 45 ± 4; *p* < 0.1) (Fig. 3b). ASL
405 treatments also had no effect on the modal size distribution of the polyps in comparison to vehicle-only treatment
406 (vehicle- 4 mm vs ASL 4 mm; *p* > 0.05) (Fig. 3c).

407 No significant difference in hematocrit level were observed in *wt* mice fed with either vehicle-only
408 control or 50 mg kg⁻¹ ASL for 35 or 70 days (*p* = 0.8132 and *p* = 0.9685 respectively) (Fig. 3d and Fig. 3e).
409 Hematocrit levels in *wt* mice were also significantly higher than those of *Apc*^{min+/-} mice, irrespective of treatment
410 with either vehicle-only control or ASL (*p* = > 0.05) (Fig. 3d and Fig. 3e). After 35 days feeding with 50 mg kg⁻¹
411 ASL, no significant differences in haematocrit levels were observed in *Apc*^{min+/-} mice compared to those fed with
412 vehicle-only control (vehicle-only = 41.2 ± 0.8 vs ASL = 40.0 ± 1.1; *p* = 0.9906) (Fig. 3d). However, following
413 70 days of administration with 50 mg kg⁻¹ ASL, haematocrit levels were found to be significantly higher in

414 *Apc*^{min/+} mice fed in comparison to those fed with the vehicle-only control (vehicle-only = 36.0 ± 0.9 vs ASL =
415 42 ± 1.9; *p* = 0.012) (Fig. 3e).

416

417 ASL effects the splenic weight, proportion of spleen red pulp in *Apc*^{min/+} mice

418

419 The spleens from vehicle-only control fed *Apc*^{min/+} mice were significantly heavier than their *wt* littermates (0.58
420 ± 0.2g vs 0.15g ± 0.5g; *p* < 0.0001). A dose of 50 mg kg⁻¹ ASL had no effect on splenic weight in *wt* mice after
421 70 days of treatment. However, administration of 50 mg kg⁻¹ ASL for 70 days to *Apc*^{min/+} mice resulted in a
422 statistically significant decrease in splenic weight (0.58 ± 0.2 g vs 0.40 ± 0.4g; *p* < 0.001) (Fig. 4a). Following
423 treatment of the *Apc*^{min/+} mice with 50 mg kg⁻¹ ASL there was a significant reduction in the proportion of red pulp
424 as compared with vehicle-only controls (62 ± 3.2 vs 48 ± 1.4; *p* < 0.001), no significant difference was observed
425 in the *wt* mice (Fig. 4b). Histological examination of sections from *wt* mouse spleen revealed conspicuous and
426 intensely basophilic areas of white pulp; these were separated by less dense regions of red pulp; areas that are
427 responsible for removal of old or damaged erythrocytes. In vehicle-only control treated *Apc*^{min/+} mice there was
428 obvious clumping of the red pulp, and the proportion of this tissue was significantly increased when compared to
429 *wt* mice (Fig. 4c). *Apc*^{min/+} mice fed with ASL showed reduced clumping of red pulp and an increase in the white
430 pulp regions, like that of the *wt* mice (Fig. 4c).

431

432 **Discussion**

433

434 The ability of chemotherapeutic agents to selectively target cancerous cells while sparing normal tissue is a highly
435 desirable trait, as it may mitigate against the common side effects associated with these toxic therapeutics such as
436 epithelial cell damage in the gastrointestinal tract, immunosuppression (in the bone marrow) and hair loss (Carey
437 and Burish 1988; McQuade et al. 2014). Biosurfactant compounds such as sophorolipids are naturally produced
438 agents that may possess the ability to differentially affect cancer and normal epithelial cells and as such their anti-
439 cancer activities are a growing area of research. Here we investigated the *in vitro* effects of a 94% pure preparation
440 of ASL on a non-transformed intestinal epithelial cell line (CCD-841-CoN) and 4 colorectal cancer cell lines
441 (HT29, HT115, Caco2, and HCT116). Colonic epithelia (CCD-841-CoN) treated with ASL showed no sign of
442 toxicity as evidenced by maintenance of their viability at doses ranging between 0.001 and 100 µg mL⁻¹. However,
443 at doses of 20 µg mL⁻¹ and above, ASL's potently reduced cell viability in all the colorectal cancer cell lines

444 examined. Although the cytotoxic potency of sophorolipid mixtures has been reported against cancer cell lines
445 previously, (Chen et al. 2006b; Fu et al. 2008; Shao et al. 2012; Ribeiro et al. 2015; Callaghan et al. 2016; Adu et
446 al. 2022), this is the first time an anti-cancer effect has been reported from a purified and well characterised ASL
447 on a range of cell lines from the same tissue of origin (i.e. colorectal cancer cells).

448 Importantly, the ASL preparation utilised here exhibited a differential effect *in vitro* on non-transformed
449 cell lines compared to CRC cells, with a dose-dependent cytotoxic response in all five CRC cell lines. A
450 differential effect has been reported in previous studies where SL were cytotoxic to pancreatic, liver and melanoma
451 cancer cell lines but with no demonstrated toxicity to non-transformed cell lines (Fu et al. 2008; Adu et al. 2022).
452 However, Fu *et al.* 2008 utilised non-adherent circulating blood monocytes (PBMC) as a control for adherent
453 transformed cells making conclusions from this study difficult to interpret. Therefore, other than Adu and
454 colleagues work on melanoma cell lines, a directly comparable description of the specificity of a pure preparation
455 of ASL that uses a normal adherent cell line from the same tissue of origin has not been carried out.

456 It has been hypothesised that SL can intercalate into the cytoskeleton of cells resulting in membrane
457 disruption. Changes in cell morphology consistent with disruption of membrane cytoskeletal protein distribution
458 have previously been shown to occur with SL doses as low as 30 $\mu\text{g mL}^{-1}$ in pancreatic H7402 and lung cancer
459 A549 cell lines (Chen et al. 2006b). Our study demonstrated that a preparation dominated by a single congener of
460 ASL at low concentrations (40 $\mu\text{g mL}^{-1}$) induced cell rounding, cytoplasmic condensation and cell detachment in
461 all the CRC cancer cell lines tested, with the adenocarcinoma cell lines HT29 and Caco2 being most susceptible.
462 At doses of 70 – 100 $\mu\text{g mL}^{-1}$, ASL caused a significant increase in the numbers of detached cells with all cells in
463 the supernatant from treated cultures showing morphological features consistent with apoptosis or necrosis.
464 Similar results have been observed in HepG2 liver cancer cells treated with LSL preparations (Wang et al. 2021).

465 ASL's used at the doses described in our study, induced a reduction of tension at the interfacial region
466 of the bilayer resulting in phospholipid dehydration which affected lipid stability and ultimately resulted in cell
467 death (Maget-Dana et al. 1989; Shah et al. 2005; Ortiz et al. 2009). To assess cell death mechanisms caused by
468 ASL's, ethidium bromide/acridine orange staining was carried out allowing the morphological identification of
469 the type of cell death. We have conclusively demonstrated that ASL induces both apoptosis and necrosis in these
470 cancer cell lines in a dose-dependent manner. The ability of ASL to induce either apoptosis or necrosis may be
471 cell-line specific, as high proportions of apoptotic cells have been reported following the addition of diacetylated
472 LSL to liver (H7402) and lung cancer (H7402) cell cultures, while necrosis is primarily observed in a pancreatic

473 carcinoma cell line (HPAC) treated with sophorolipid mixtures, LSL or methyl ester derivative SL (Shao et al.
474 2012).

475 An important characteristic of malignant growth is the ability of tumour cells to leave their restricted
476 compartment and gain access to blood vessels to initiate the first phase of metastasis (Hanahan and Weinberg
477 2011). The movement of cells across tissues therefore plays an important role in this progression thus highlighting
478 the need for an agent that can counteract the migratory and diapedesis properties of colorectal cancer cells
479 (Dianzani et al. 2014). It has been postulated that the amphiphilic properties of sophorolipids permit their
480 incorporation into the mammalian cellular membrane disrupting cellular functions such as proliferation and
481 migration (Zhao et al. 2013; Haque et al. 2021; Adu et al. 2022). We assessed the ability of ASL's to inhibit
482 migration of the CRC cell lines HT29 and HT115. At a low dose ($10 \mu\text{g mL}^{-1}$) of ASL there was no effect on
483 normal colonic epithelial cells (CCD-841-CoN) and scratch coverage after 72 hours. However, the same dose of
484 ASL applied to colorectal cancer cell lines HT29 and HT115 cultures reduced the total percentage area of scratch
485 covered to between 12-25%. An inhibition of migration induced by sophorolipid (as measured in the scratch assay)
486 has been documented. In 2015, Riberiro and co-workers showed that $5 \mu\text{g mL}^{-1}$ of a 93% pure, diacetylated LSL
487 resulted in significantly reduced migration of MDA-MB-231 breast cancer cells (Ribeiro et al. 2015), although to
488 our knowledge no reports are available for a study that solely utilises purified ASL. Adu *et al.* 2022 showed that
489 the migration of a melanoma cell line SK-MEL-28 was significantly reduced when treated with LSL and ASL
490 preparations in comparison with cells treated with a vehicle-only control, and immortalized keratinocytes (HaCaT)
491 treated with the same sophorolipid preparations (Adu et al. 2022). The use of both migration and invasion assays
492 incorporating the Boyden chamber are widely used to test candidate chemotherapeutics with the HT29 and HT115
493 cell lines commonly employed to test anti-invasive properties of potential chemotherapeutics *in vitro* (Li and Zhu
494 1999). Currently the only studies investigating the potential anti-metastatic properties of biosurfactants are limited
495 to the inhibitory effects on breast cancer cell lines MCF and MDA-MB-231 *in vitro* invasion mediated by the
496 lipopeptide Surfactin produced by *Bacillus subtilis* (Park et al. 2013). Addition of $10 \mu\text{M}$ (equivalent to approx.
497 $660 \mu\text{g mL}^{-1}$ of ASL in this study) of Surfactin had the ability to reduce the migration of MCF-7 and MDA-MB-
498 231 through an extracellular matrix by 68% and 84% respectively (Park et al. 2013). The Surfactin used by Park
499 *et al.* (2013) also reduced the colony forming ability of both cell lines by 70% and 61%. To investigate the
500 migration inhibitory properties of ASL, HT29 and HT115 cells were plated on a porous membrane and allowed
501 to migrate through a septum in response to a FBS stimulus in the chamber below. ASL had no effect on the
502 migration of normal colonic CCD-841-CoN cells, however following treatment with $10 \mu\text{g mL}^{-1}$ and $50 \mu\text{g mL}^{-1}$

503 ASL, migration was significantly decreased in HT29 cell lines by 65% and 72% respectively, and 86% and 81%
504 in HT115 cell lines.

505 The anti-tumour activities of sophorolipid preparations *in vivo* are unclear. In a study by Li and co-
506 workers, an intragastric administered mixture composed of 6 di-acetylated LSL congeners was shown to reduce
507 the size of tumours in a murine HeLa xenograft model (Li et al. 2017). Whereas a purified form of LSL
508 exaggerated the growth of neoplasm along the intestinal tract and increased intestinal blood loss in the $Apc^{min/+}$ -
509 mouse (Callaghan et al. 2016). In this study, we use the $Apc^{min/+}$ mouse model of FAP, to evaluate the
510 chemotherapeutic potential of purified ASL in the treatment of solid neoplasms. $Apc^{min/+}$ mice develop intestinal
511 adenomatous neoplasms (polyps), and animals typically present with enlarged spleens and reduced haematocrit
512 by 4 months of age (Yekkala and Baudino 2007). This is an acute model with a life span of <150 days, where the
513 primary cause of death is not directly attributable to the development of numerous polyps but rather as a result of
514 extensive intestinal bleeding and anaemia (Hinoi et al. 2007). When administered orally, topically or via *i.v.*
515 injection sophorolipid mixtures are well tolerated (Ikeda et al. 1986). Similarly, in our study, both *wt* and $Apc^{min/+}$ -
516 mice tolerated the oral administration of ASL with no measurable gross anatomical or behavioural differences
517 noted. Post-mortem analysis also revealed no effects on gross measures of peritoneal organs (size and dimensions)
518 after ASL treatment. However, oral feeding of ASL to $Apc^{min/+}$ for 70 days resulted in a reduction of spleen size
519 and a significant increase in haematocrit, consistent with decreased intestinal bleeding and improvement in the
520 associated anaemia characteristic of this model (Perkins et al. 2002; Hinoi et al. 2007). This is a potentially
521 significant finding, as rectal bleeding and anaemia are reported in over 30% of CRC patients and it is a contributing
522 factor in reduced lifespan in both humans as well as $Apc^{min/+}$ mice (Ronnekleiv-Kelly and Kennedy 2011). In
523 humans, laser ablation encourages coagulation of tumours demonstrating significant blood loss and this technique
524 has shown to be effective after 2 - 5 treatments with a success rate of 80% (Kimmey 2004). However recurrent
525 bleeding episodes result in surgical intervention in 2 - 15% of patients (Rao et al. 2005). The oral administration
526 of a well-tolerated non-toxic, pro-thrombotic agents to reduce intestinal blood loss in patients with haemorrhagic
527 colorectal tumours may be a useful addition to the therapeutic treatment of these conditions.

528 In conclusion, the purified ASL mixture we utilised in this study differentially affects non-transformed
529 in comparison to colorectal cancer cell lines, resulting in a significant and dose-dependent decrease in their
530 viability, migration, and anchorage-independent growth characteristics. While ASL does not change either the
531 size or number of intestinal polyps in $Apc^{min/+}$ mice, both spleen size and tumour-associated bleeding was reduced.

532 This warrants further investigation of this orally available biosurfactant as a chemotherapeutic for delaying disease
533 progression in pre-cancerous colorectal neoplasms.

534

535 **Author Contributions**

536

537 BC and MST contributed equally to this work. INAVB, RM, CM and IMB conceived and designed the research.

538 BC and NB conducted experiments. BC and MST analysed data and wrote the manuscript. All authors read and

539 approved the manuscript.

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544 **References**

545

546 Adu SA, Naughton PJ, Marchant R, Banat IM (2020) Microbial Biosurfactants in Cosmetic and Personal Skincare
547 Pharmaceutical Formulations. *Pharmaceutics* 12:1099

548 Adu SA, Twigg MS, Naughton PJ, Marchant R, Banat IM (2022) Biosurfactants as Anticancer Agents:
549 Glycolipids Affect Skin Cells in a Differential Manner Dependent on Chemical Structure. *Pharm.* 2022,
550 Vol. 14, Page 360 14:360

551 Altman SA, Randers L, Rao G (1993) Comparison of Trypan Blue Dye Exclusion and Fluorometric Assays for
552 Mammalian Cell Viability Determinations. *Biotechnol Prog* 9:671–674.
553 <https://doi.org/10.1021/bp00024a017>

554 Baccile N, Cuvier AS, Prévost S, Stevens C V., Delbeke E, Berton J, Soetaert W, Van Bogaert INA, Roelants S
555 (2016) Self-Assembly Mechanism of pH-Responsive Glycolipids: Micelles, Fibers, Vesicles, and Bilayers.
556 *Langmuir* 32:10881–10894. <https://doi.org/10.1021/acs.langmuir.6b02337>

557 Baccile N, Cuvier AS, Valotteau C, Van Bogaert INA (2013) Practical methods to reduce impurities for gram-
558 scale amounts of acidic sophorolipid biosurfactants. *Eur J Lipid Sci Technol* 115:1404–1412.
559 <https://doi.org/10.1002/ejlt.201300131>

560 Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R (2010) Microbial
561 biosurfactants production, applications and future potential. *Appl Microbiol Biotechnol* 87:427–444.

562 <https://doi.org/10.1007/s00253-010-2589-0>

563 Barbuti AM, Chen ZS (2015) Paclitaxel through the ages of anticancer therapy: Exploring its role in
564 chemoresistance and radiation therapy. *Cancers (Basel)*. 7:2360–2371

565 Beutler JA (2019) Natural Products as a Foundation for Drug Discovery. *Curr. Protoc. Pharmacol.* 86:e67

566 Bluth M, Fu S, Fu A, Stanek A, Smithnorowitz T, Wallner S, Gross R, Nowakowski M, Zenilman M (2008)
567 Sophorolipids Decrease Asthma Severity And Ova-specific IgE Production In A Mouse Asthma Model. *J*
568 *Allergy Clin Immunol* 121:S2–S2. <https://doi.org/10.1016/j.jaci.2007.12.011>

569 Callaghan B, Lydon H, Roelants SLKW, Van Bogaert INA, Marchant R, Banat IM, Mitchell CA (2016) Lactonic
570 Sophorolipids Increase Tumor Burden in *Apc^{min+/-}* Mice. *PLoS One* 11:e0156845

571 Carey MP, Burish TG (1988) Etiology and Treatment of the Psychological Side Effects Associated With Cancer
572 Chemotherapy: A Critical Review and Discussion. *Psychol Bull* 104:307–325.
573 <https://doi.org/10.1037/0033-2909.104.3.307>

574 Casas J, García-Ochoa F (1999) Sophorolipid production by *Candida bombicola*: Medium composition and
575 culture methods. *J Biosci Bioeng* 88:488–494. [https://doi.org/https://doi.org/10.1016/S1389-](https://doi.org/https://doi.org/10.1016/S1389-1723(00)87664-1)
576 [1723\(00\)87664-1](https://doi.org/https://doi.org/10.1016/S1389-1723(00)87664-1)

577 Casteleyn C, Doom M, Lambrechts E, van den Broeck W, Simoens P, Cornillie P (2010) Locations of gut-
578 associated lymphoid tissue in the 3-month-old chicken: A review. *Avian Pathol* 39:143–150.
579 <https://doi.org/10.1080/03079451003786105>

580 Ceresa C, Fracchia L, Fedeli E, Porta C, Banat IM (2021) Recent advances in biomedical, therapeutic and
581 pharmaceutical applications of microbial surfactants. *Pharmaceutics* 13:466.
582 <https://doi.org/10.3390/pharmaceutics13040466>

583 Chen HC (2005) Boyden chamber assay. In: Guan J-L (ed) *Methods in molecular biology* (Clifton, N.J.). Humana
584 Press, Totowa, NJ, pp 15–22

585 Chen J, Song X, Zhang H, Qu Y (2006a) Production, structure elucidation and anticancer properties of
586 sophorolipid from *Wickerhamiella domercqiae*. *Enzyme Microb Technol* 39:501–506.
587 <https://doi.org/https://doi.org/10.1016/j.enzmictec.2005.12.022>

588 Chen J, Song X, Zhang H, Qu YB, Miao JY (2006b) Sophorolipid produced from the new yeast strain
589 *Wickerhamiella domercqiae* induces apoptosis in H7402 human liver cancer cells. *Appl Microbiol*
590 *Biotechnol* 72:52–59. <https://doi.org/10.1007/s00253-005-0243-z>

591 D’Incalci M, Steward WP, Gescher AJ (2005) Use of cancer chemopreventive phytochemicals as antineoplastic

592 agents. *Lancet Oncol* 6:899–904. [https://doi.org/10.1016/s1470-2045\(05\)70425-3](https://doi.org/10.1016/s1470-2045(05)70425-3)

593 Dhar S, Reddy EM, Prabhune A, Pokharkar V, Shiras A, Prasad BL V (2011) Cytotoxicity of sophorolipid-gellan
594 gum-gold nanoparticle conjugates and their doxorubicin loaded derivatives towards human glioma and
595 human glioma stem cell lines. *Nanoscale* 3:575–580. <https://doi.org/10.1039/C0NR00598C>

596 Dianzani C, Minelli R, Gigliotti CL, Occhipinti S, Giovarelli M, Conti L, Boggio E, Shivakumar Y, Baldanzi G,
597 Malacarne V, Orilieri E, Cappellano G, Fantozzi R, Sblattero D, Yagi J, Rojo JM, Chiocchetti A, Dianzani
598 U (2014) B7h Triggering Inhibits the Migration of Tumor Cell Lines. *J Immunol* 192:4921–4931.
599 <https://doi.org/10.4049/jimmunol.1300587>

600 Elshikh M, Funston S, Chebbi A, Ahmed S, Marchant R, Banat IM (2017) Rhamnolipids from non-pathogenic
601 *Burkholderia thailandensis* E264: Physicochemical characterization, antimicrobial and antibiofilm efficacy
602 against oral hygiene related pathogens. *N Biotechnol* 36:26–36. <https://doi.org/10.1016/j.nbt.2016.12.009>

603 Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F (2020) Global
604 Cancer Observatory: Cancer Today. <https://gco.iarc.fr/today>. Accessed 3 Dec 2021

605 Flam F (1994) Race to synthesize taxol ends in a tie. *Science* (80-.). 263:911

606 Fridlender M, Kapulnik Y, Koltai H (2015) Plant derived substances with anti-cancer activity: From folklore to
607 practice. *Front Plant Sci* 6:799. <https://doi.org/10.3389/fpls.2015.00799>

608 Fu SL, Wallner SR, Bowne WB, Hagler MD, Zenilman ME, Gross R, Bluth MH (2008) Sophorolipids and Their
609 Derivatives Are Lethal Against Human Pancreatic Cancer Cells. *J Surg Res* 148:77.
610 <https://doi.org/10.1016/j.jss.2008.03.005>

611 Hanahan D, Weinberg RA (2011) Hallmarks of cancer: The next generation. *Cell* 144:646–674

612 Haque F, Khan MSA, AlQurashi N (2021) ROS-Mediated Necrosis by Glycolipid Biosurfactants on Lung, Breast,
613 and Skin Melanoma Cells. *Front. Oncol.* 11:253

614 Hardin R, Pierre J, Schulze R, Mueller CM, Fu SL, Wallner SR, Stanek A, Shah V, Gross RA, Weedon J,
615 Nowakowski M, Zenilman ME, Bluth MH (2007) Sophorolipids Improve Sepsis Survival: Effects of Dosing
616 and Derivatives. *J Surg Res* 142:314–319. <https://doi.org/10.1016/j.jss.2007.04.025>

617 Hinoi T, Akyol A, Theisen BK, Ferguson DO, Greenson JK, Williams BO, Cho KR, Fearon ER (2007) Mouse
618 model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. *Cancer Res* 67:9721–
619 9730. <https://doi.org/10.1158/0008-5472.CAN-07-2735>

620 Ikeda Y, Sunakawa T, Tsuchiya S, Kondo M, Okamoto K (1986) Toxicological studies on sophorolipid
621 derivatives. (I) Acute toxicity, eye irritation, primary skin irritation, skin sensitization, phototoxicity,

622 photosensitization, mutagenicity of polyoxypropylene (12) [(2'-0-β-D-glucopyranosyl-β-D-
623 glucopyranosyl)oxy-]. J Toxicol Sci 11:197–211. <https://doi.org/10.2131/jts.11.197>

624 Kelloff GJ, Boone CW, Crowell JA, Steele VE, Lubet R, Sigman CC (1994) Chemopreventive drug development:
625 perspectives and progress. Cancer Epidemiol biomarkers Prev a Publ Am Assoc Cancer Res cosponsored
626 by Am Soc Prev Oncol 3:85–98

627 Khalifa SAM, Elias N, Farag MA, Chen L, Saeed A, Hegazy MEF, Moustafa MS, El-Wahed AA, Al-Mousawi
628 SM, Musharraf SG, Chang FR, Iwasaki A, Suenaga K, Alajlani M, Göransson U, El-Seedi HR (2019)
629 Marine natural products: A source of novel anticancer drugs. Mar Drugs 17:491.
630 <https://doi.org/10.3390/md17090491>

631 Kimmey MB (2004) Endoscopic methods (other than stents) for palliation of rectal carcinoma. J Gastrointest Surg
632 8:270–273. <https://doi.org/10.1016/j.gassur.2003.11.017>

633 Li H, Guo W, Ma X, Li J, Song X (2017) *In Vitro* and *In Vivo* Anticancer Activity of Sophorolipids to Human
634 Cervical Cancer. Appl Biochem Biotechnol 181:1372–1387. <https://doi.org/10.1007/s12010-016-2290-6>

635 Li YH, Zhu C (1999) A modified Boyden chamber assay for tumor cell transendothelial migration *in vitro*. Clin
636 Exp Metastasis 17:423–429. <https://doi.org/10.1023/A:1006614232388>

637 Maget-Dana R, Harnois I, Ptak M (1989) Interactions of the lipopeptide antifungal iturin A with lipids in mixed
638 monolayers. BBA - Biomembr 981:309–314. [https://doi.org/10.1016/0005-2736\(89\)90042-4](https://doi.org/10.1016/0005-2736(89)90042-4)

639 Marchant R, Banat IM (2012a) Biosurfactants: A sustainable replacement for chemical surfactants? Biotechnol
640 Lett 34:1597–1605. <https://doi.org/10.1007/s10529-012-0956-x>

641 Marchant R, Banat IM (2012b) Microbial biosurfactants: Challenges and opportunities for future exploitation.
642 Trends Biotechnol. 30:558–565

643 Mazevet M, Moulin M, Llach-Martinez A, Chargari C, Deutsch É, Gomez A-M, Morel É (2013) Complications
644 of chemotherapy, a basic science update. Presse Med 42:e352–e361.
645 <https://doi.org/https://doi.org/10.1016/j.lpm.2013.06.011>

646 McQuade RM, Bornstein JC, Nurgali K (2014) Anti-Colorectal Cancer Chemotherapy-Induced Diarrhoea:
647 Current Treatments and Side-Effects. Int J Clin Med 05:393–406. <https://doi.org/10.4236/ijcm.2014.57054>

648 Morgan MA, Rubin SC (1998) Long-term complications of chemotherapy. Prim Care Update Ob Gyns 5:65–68.
649 [https://doi.org/https://doi.org/10.1016/S1068-607X\(98\)00010-9](https://doi.org/https://doi.org/10.1016/S1068-607X(98)00010-9)

650 Moser AR, Luongo C, Gould KA, McNeley MK, Shoemaker AR, Dove WF (1995) ApcMin: A mouse model for
651 intestinal and mammary tumorigenesis. Eur J Cancer 31:1061–1064. [22](https://doi.org/10.1016/0959-</p></div><div data-bbox=)

652 8049(95)00181-H

653 Naughton PJ, Marchant R, Naughton V, Banat IM (2019) Microbial biosurfactants: current trends and applications
654 in agricultural and biomedical industries. *J Appl Microbiol* 127:12–28. <https://doi.org/10.1111/jam.14243>

655 O’Connell MJ, Lavery I, Yothers G, Paik S, Clark-Langone KM, Lopatin M, Watson D, Baehner FL, Shak S,
656 Baker J, Cowens JW, Wolmark N (2010) Relationship Between Tumor Gene Expression and Recurrence
657 in Four Independent Studies of Patients With Stage II/III Colon Cancer Treated With Surgery Alone or
658 Surgery Plus Adjuvant Fluorouracil Plus Leucovorin. *J Clin Oncol* 28:3937–3944.
659 <https://doi.org/10.1200/JCO.2010.28.9538>

660 Ortiz A, Teruel JA, Espuny MJ, Marqués A, Manresa Á, Aranda FJ (2009) Interactions of a bacterial biosurfactant
661 trehalose lipid with phosphatidylserine membranes. *Chem Phys Lipids* 158:46–53.
662 <https://doi.org/10.1016/j.chemphyslip.2008.11.001>

663 Park SY, Kim JH, Lee YJ, Lee SJ, Kim Y (2013) Surfactin suppresses TPA-induced breast cancer cell invasion
664 through the inhibition of MMP-9 expression. *Int J Oncol* 42:287–296. <https://doi.org/10.3892/ijo.2012.1695>

665 Perkins S, Verschoyle RD, Hill K, Sharma RA, Williams ML, Steward WP, Gescher AJ, Parveen I, Threadgill
666 MD (2002) Chemopreventive efficacy and pharmacokinetics of curcumin in the min/+ mouse, a model of
667 familial adenomatous polyposis. *Cancer Epidemiol Biomarkers Prev* 11:535–540

668 Rao VSR, Al-Mukhtar A, Rayan F, Stojkovic S, Moore PJ, Ahmad SM (2005) Endoscopic laser ablation of
669 advanced rectal carcinoma - A DGH experience. *Color. Dis.* 7:58–60

670 Reuter BK, Zhang X-J, Miller MJS (2002) Therapeutic utility of aspirin in the Apc^{Min/+} murine model of colon
671 carcinogenesis. *BMC Cancer* 2:19. <https://doi.org/10.1186/1471-2407-2-19>

672 Ribeiro IAC, Faustino CMC, Guerreiro PS, Frade RFM, Bronze MR, Castro MF, Ribeiro MHL (2015)
673 Development of novel sophorolipids with improved cytotoxic activity toward MDA-MB-231 breast cancer
674 cells. *J Mol Recognit* 28:155. <https://doi.org/10.1002/jmr.2403>

675 Roelants SLKW, Ciesielska K, De Maeseneire SL, Moens H, Everaert B, Verweire S, Denon Q, Vanlerberghe B,
676 Van Bogaert INA, Van der Meeren P, Devreese B, Soetaert W (2016) Towards the industrialization of new
677 biosurfactants: Biotechnological opportunities for the lactone esterase gene from *Starmarella bombicola*.
678 *Biotechnol Bioeng* 113:550–559. <https://doi.org/10.1002/bit.25815>

679 Roelants SLKW, Solaiman DKY, Van Renterghem L, Lodens S, Soetaert W, Ashby R (2019) Production and
680 application of sophorolipids. In: Hayes DG, Ashby RD, Solaiman D. (eds) *Biobased Surfactants: Synthesis,*
681 *Properties and Applications*, 2nd edn. AOCS Press, London, pp 65–119

682 Ronnekleiv-Kelly SM, Kennedy GD (2011) Management of stage IV rectal cancer: Palliative options. *World J*
683 *Gastroenterol* 17:835–847. <https://doi.org/10.3748/wjg.v17.i7.835>

684 Sarubbo LA, Silva M da GC, Durval IJB, Bezerra KGO, Ribeiro BG, Silva IA, Twigg MS, Banat IM (2022)
685 Biosurfactants: Production, Properties, Applications, Trends, and General Perspectives. *Biochem Eng J*
686 108377. <https://doi.org/10.1016/j.bej.2022.108377>

687 Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*
688 9:671–675. <https://doi.org/10.1038/nmeth.2089>

689 Shah V, Doncel GF, Seyoum T, Eaton KM, Zalenskaya I, Hagver R, Azim A, Gross R (2005) Sophorolipids,
690 microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities.
691 *Antimicrob Agents Chemother* 49:4093–4100. <https://doi.org/10.1128/AAC.49.10.4093-4100.2005>

692 Shao L, Song X, Ma X, Li H, Qu Y (2012) Bioactivities of Sophorolipid with Different Structures Against Human
693 Esophageal Cancer Cells. *J Surg Res* 173:286–291. <https://doi.org/https://doi.org/10.1016/j.jss.2010.09.013>

694 Smyth, Thomas J. P, Perfumo, A., Marchant, R., Bannat, I.M. (2010) Isolation and anlysis of low molecular
695 weight microbial glycolipids. In: *Handbook of Hydrocarbon and Lipid Microbiology*. Berlin Heidelberg,
696 pp 3705–3723

697 Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic
698 fungus of Pacific yew. *Science* (80-) 260:214–216. <https://doi.org/10.1126/science.8097061>

699 Tucker JM, Davis C, Kitchens ME, Bunni MA, Priest DG, Spencer HT, Berger FG (2002) Response to 5-
700 fluorouracil chemotherapy is modified by dietary folic acid deficiency in *Apc^{Min/+}* mice. *Cancer Lett*
701 187:153–162. [https://doi.org/10.1016/S0304-3835\(02\)00402-0](https://doi.org/10.1016/S0304-3835(02)00402-0)

702 Twigg MS, Baccile N, Banat IM, Déziel E, Marchant R, Roelants S, Van Bogaert INA (2021) Microbial
703 biosurfactant research: time to improve the rigour in the reporting of synthesis, functional characterization
704 and process development. *Microb. Biotechnol* 14:147-170. <https://doi.org/10.1111/1751-7915.13704>

705 Van Bogaert INA, Saerens K, De Muynck C, Develter D, Soetaert W, Vandamme EJ (2007) Microbial production
706 and application of sophorolipids. *Appl Microbiol Biotechnol* 76:23–34. [https://doi.org/10.1007/s00253-](https://doi.org/10.1007/s00253-007-0988-7)
707 [007-0988-7](https://doi.org/10.1007/s00253-007-0988-7)

708 Van Bogaert INA, Zhang J, Soetaert W (2011) Microbial synthesis of sophorolipids. *Process Biochem.* 46:821–
709 833

710 Wang X, Xu N, Li Q, Chen S, Cheng H, Yang M, Jiang T, Chu J, Ma X, Yin D (2021) Lactonic sophorolipid–
711 induced apoptosis in human HepG2 cells through the Caspase-3 pathway. *Appl Microbiol Biotechnol*

712 105:2033–2042. <https://doi.org/10.1007/s00253-020-11045-5>

713 Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham DAH,
714 Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR, Eccles SA,
715 Navaratnam V, Ryder S (2010) Guidelines for the welfare and use of animals in cancer research. *Br. J.*
716 *Cancer* 102:1555–1577

717 Yekkala K, Baudino TA (2007) Inhibition of intestinal polyposis with reduced angiogenesis in *Apc^{Min/+}* mice due
718 to decreases in c-Myc expression. *Mol Cancer Res* 5:1296–1303. [https://doi.org/10.1158/1541-7786.MCR-](https://doi.org/10.1158/1541-7786.MCR-07-0232)
719 [07-0232](https://doi.org/10.1158/1541-7786.MCR-07-0232)

720 Zhao J, Wu Y, Alfred AT, Xin X, Yang S (2013) Chemical structures and biological activities of rhamnolipid
721 biosurfactants produced by *Pseudomonas aeruginosa* M14808. *J Chem Pharm Res* 5:177

722

723 **Fig. Legends**

724

725 **Fig. 1.** Treatment with ASL results in a detrimental effect on colorectal cancer cell lines *in vitro*. Compared to
726 normal colonic epithelial cells, the viability of four colorectal cancer cell lines was significantly reduced when
727 treated with ASL (a). Treatment with ASL also resulted in increased cell detachment in colorectal cancer cell lines
728 compared to normal colonic epithelial cells (b). Colorectal cancer cell lines treated with either 20 or 70 $\mu\text{g mL}^{-1}$
729 ASL showed indications of both apoptosis and necrosis driven cell death (c).

730

731 **Fig. 2.** Treatment with ASL significantly reduces cell migration in colorectal cancer cell lines. In a scratch assay
732 ASL treated colorectal cancer cells showed significantly less migration 72 h post treatment than these treated with
733 vehicle-only controls (a). Cell migration across a Boyden chamber in response to a stimulus was significantly
734 reduced in colorectal cancer cells treated with ASL in comparison vehicle-only control treated cells (b). Two-way
735 ANOVA (** $p < 0.01$ and *** $p < 0.001$).

736

737 **Fig. 3.** An ASL supplemented diet reduced intestinal bleed in a mouse mode of colorectal cancer. No
738 morphological differences were observed in ileal sections of either wt or *Apc^{min/+}* mice fed with either vehicle-
739 only controls or ASL, however vehicle-only control fed *Apc^{min/+}* mice showed evidence of intestinal bleeding
740 from polyps (arrows), whereas ASL fed *Apc^{min/+}* mice showed no evidence of intestinal bleeding (a). No
741 significant difference in intestinal polyp number (b) or polyp sizes (c) was observed in *Apc^{min/+}* mice fed on either

742 ASL or vehicle-only control. Following 35 days no significant difference was observed in haematocrit in $Apc^{min+/-}$
743 mice fed with ASL or vehicle-only control (**d**) however, after 70 days a significant increase in haematocrit in
744 $Apc^{min+/-}$ mice fed with ASL was observed compared to those fed with vehicle-only control (**e**). One-way ANOVA,
745 p values displayed on graphs.

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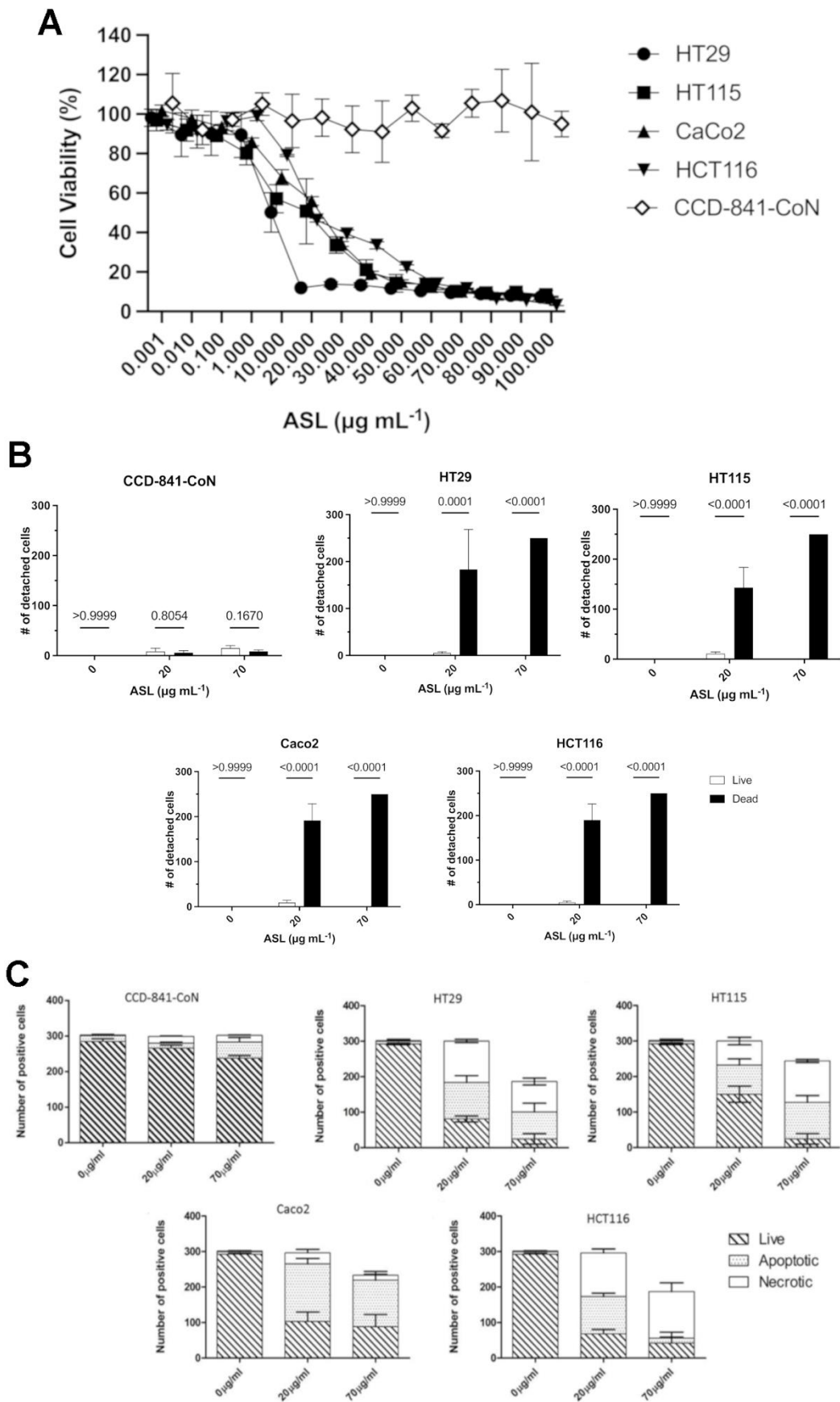
747 **Fig. 4.** Following 70 days, both splenic weight (**a**) and percentage of red pulp regions (**b**) in $Apc^{min+/-}$ mice fed
748 with ASL were significantly reduced in comparison to those fed with vehicle-only control ($p < 0.001$ and $p < 0.05$
749 respectively). $Apc^{min+/-}$ mice fed with vehicle-only control had increased red pulp with a loss reticular structure
750 compared to wild-type mice. Splenic morphology (particularly in white pulp) after feeding $Apc^{min+/-}$ mice with
751 ASL more closely resembled that seen in wild-type mice (**c**).

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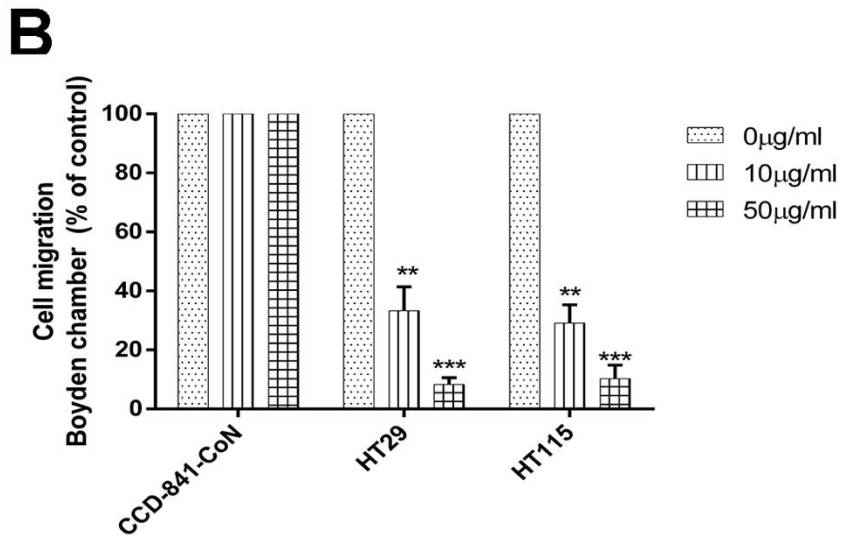
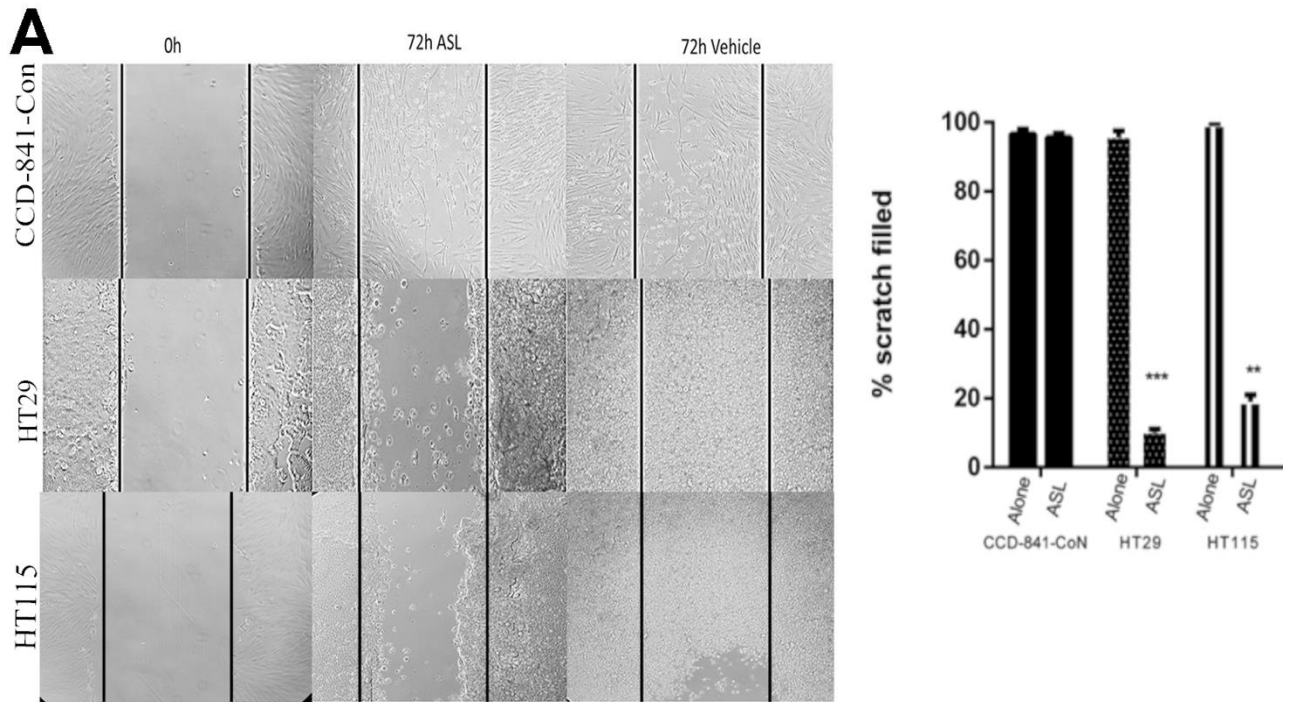
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755 Fig. 1.

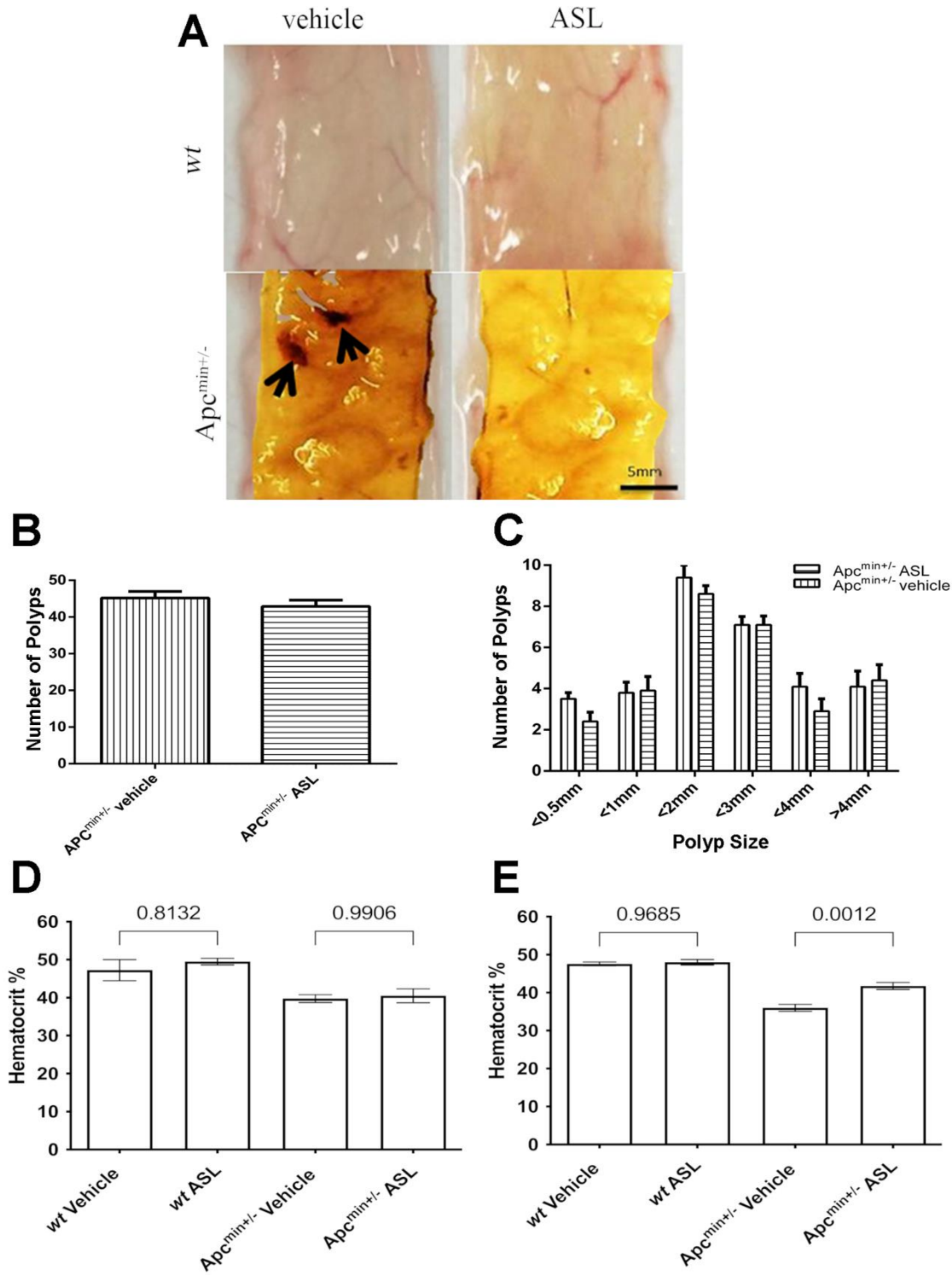


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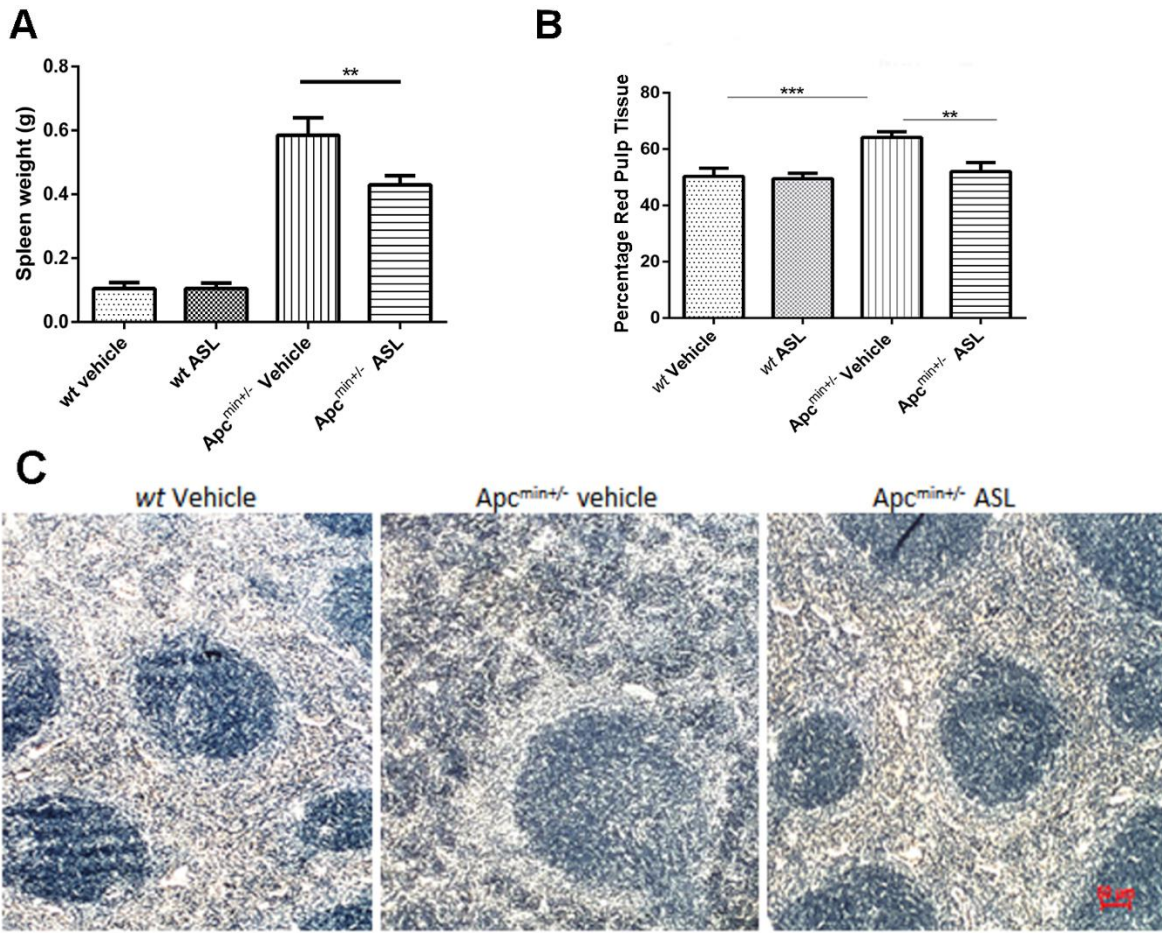
757 **Fig. 2.**



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Cell Death.tif

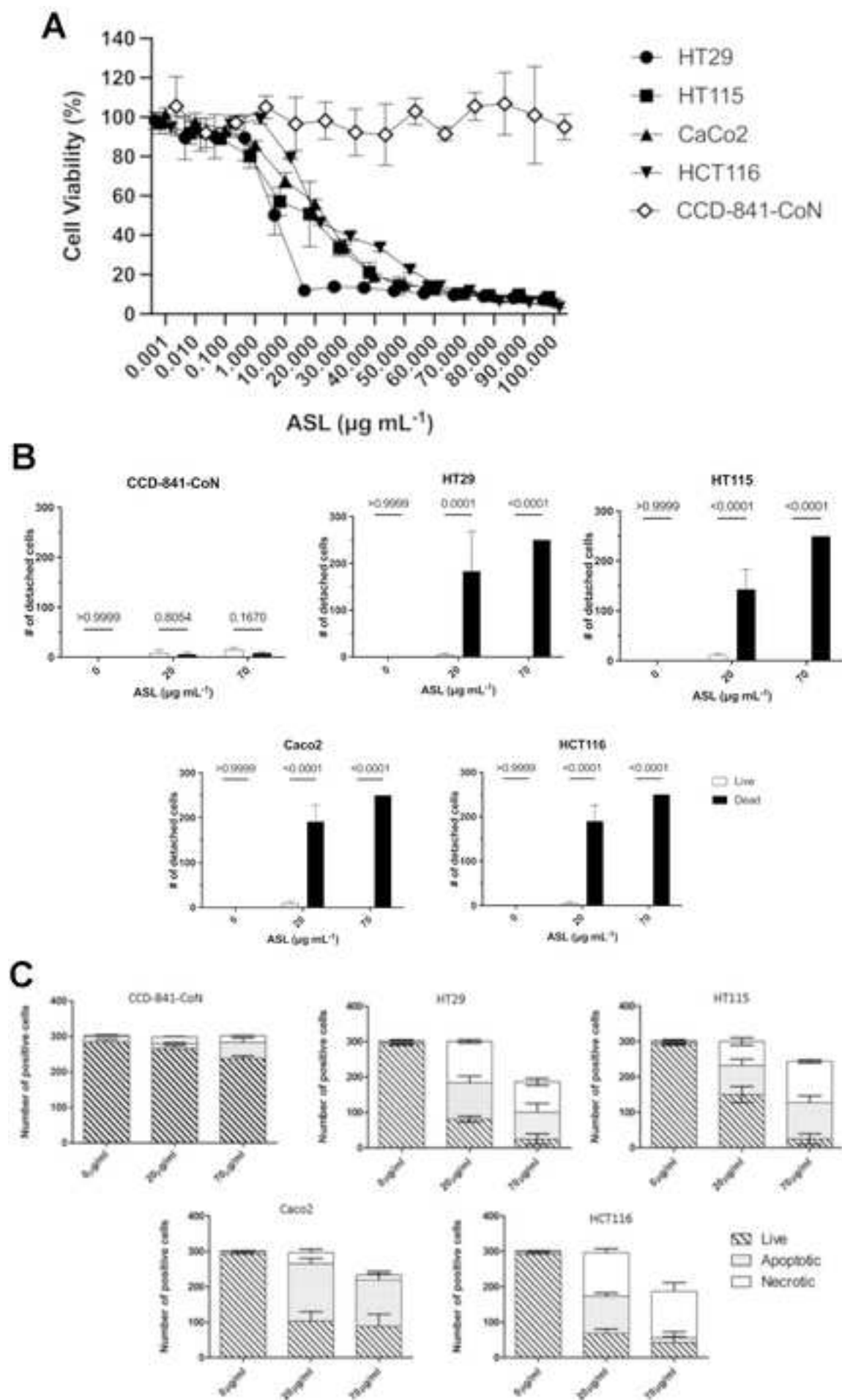


Fig2b_BoydenChamber

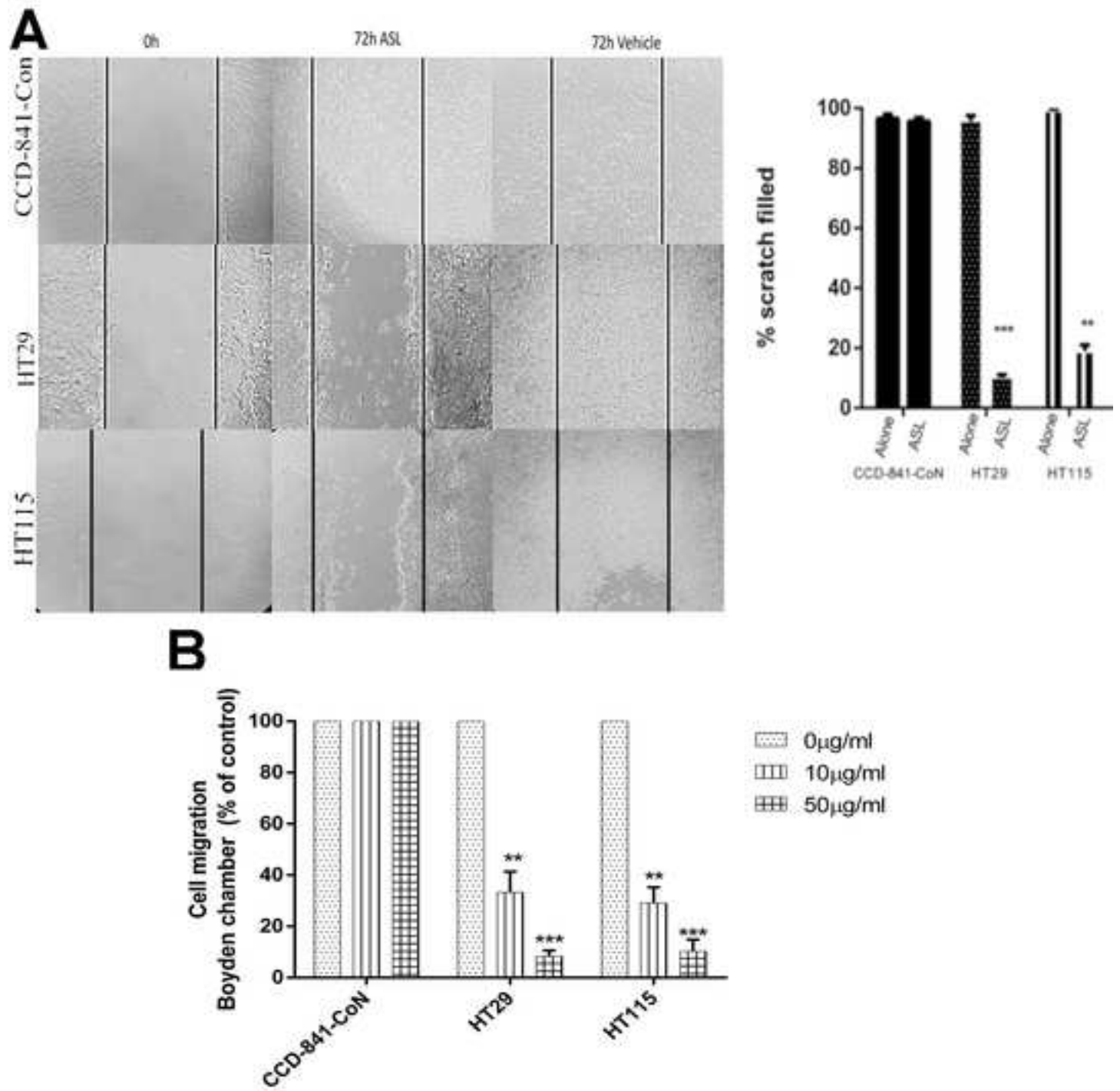
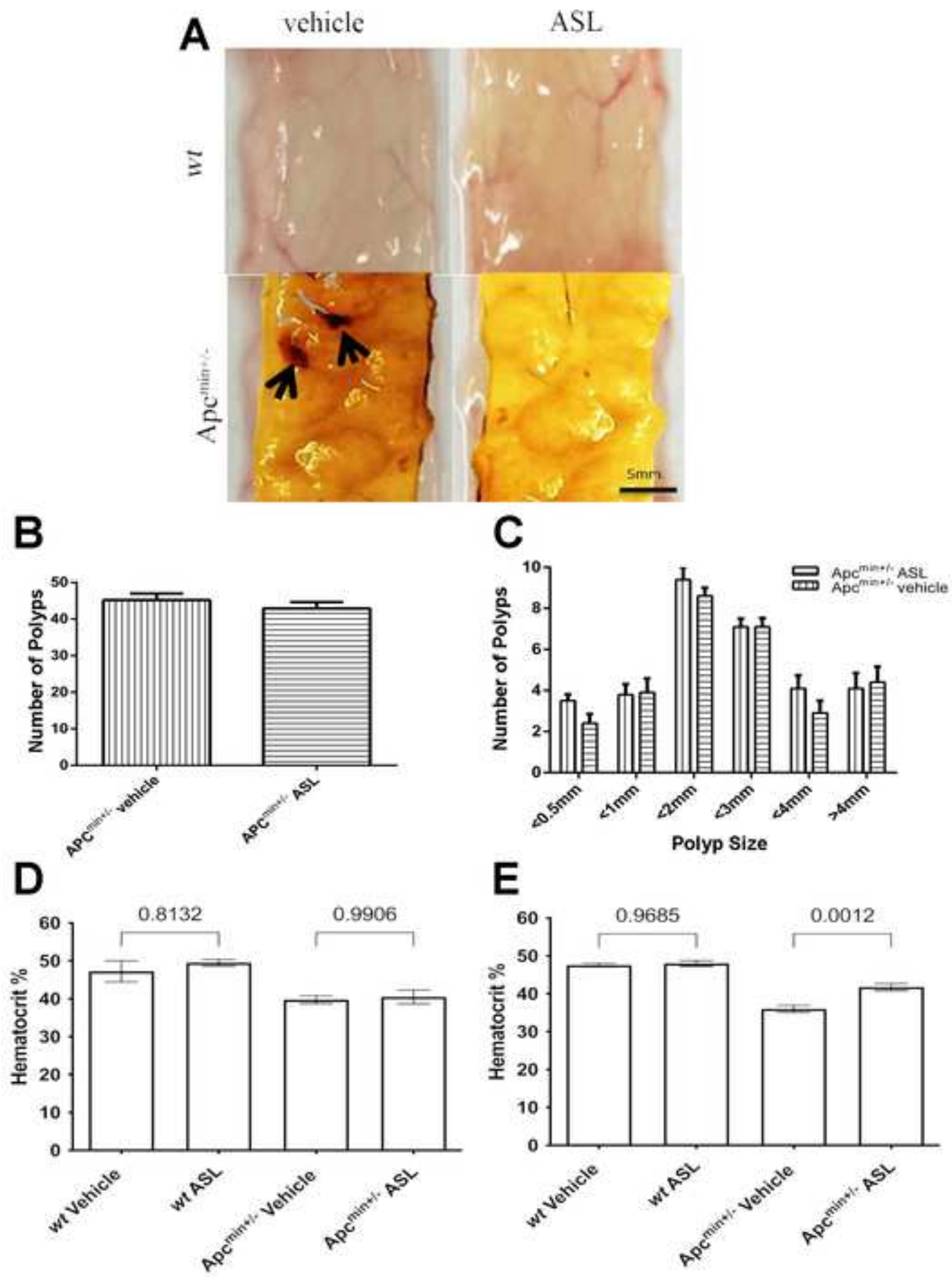
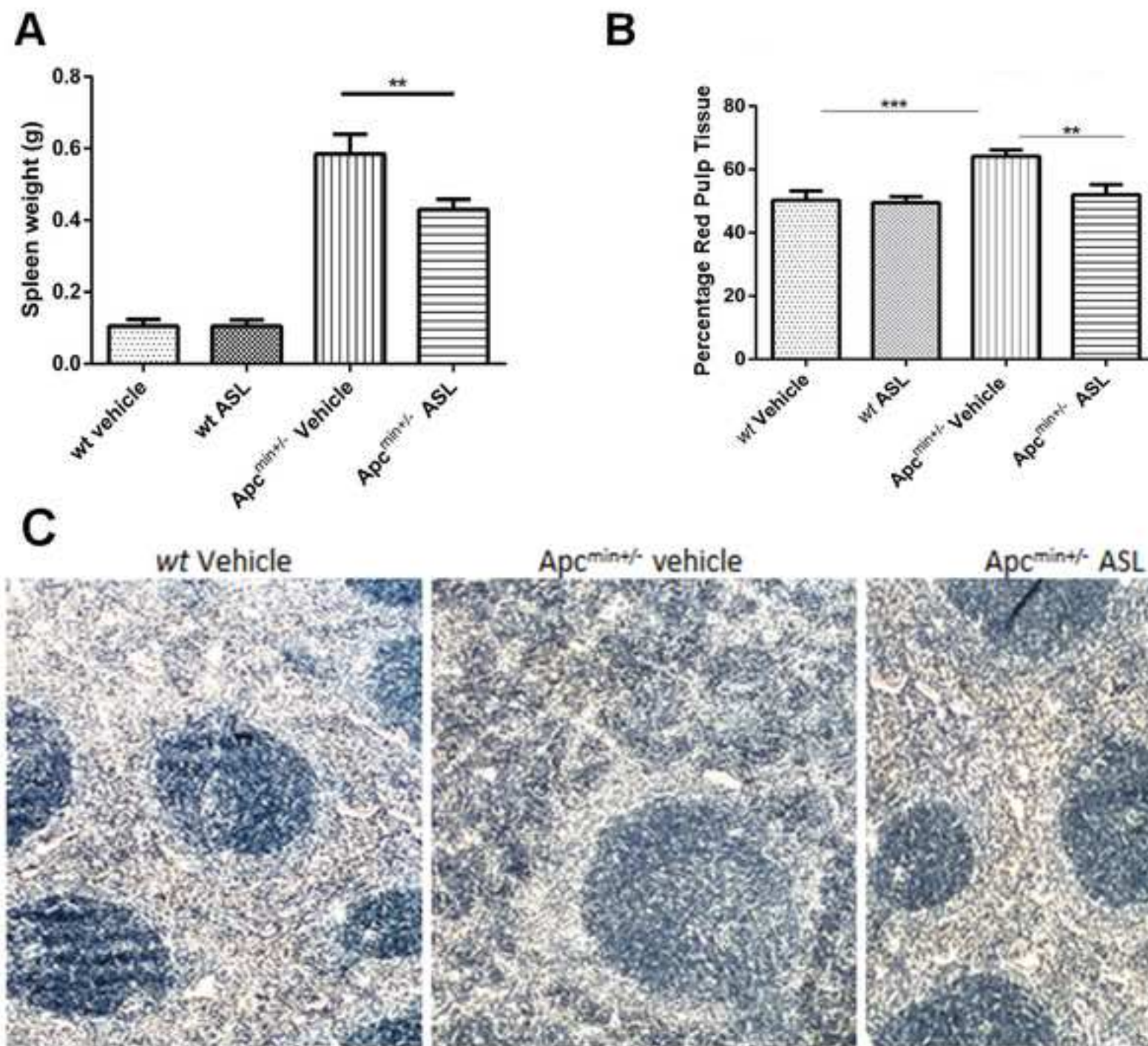
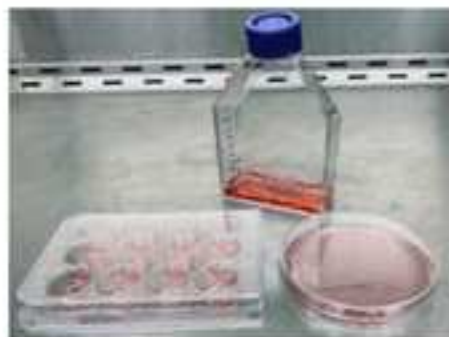


Fig3c_PolypSize





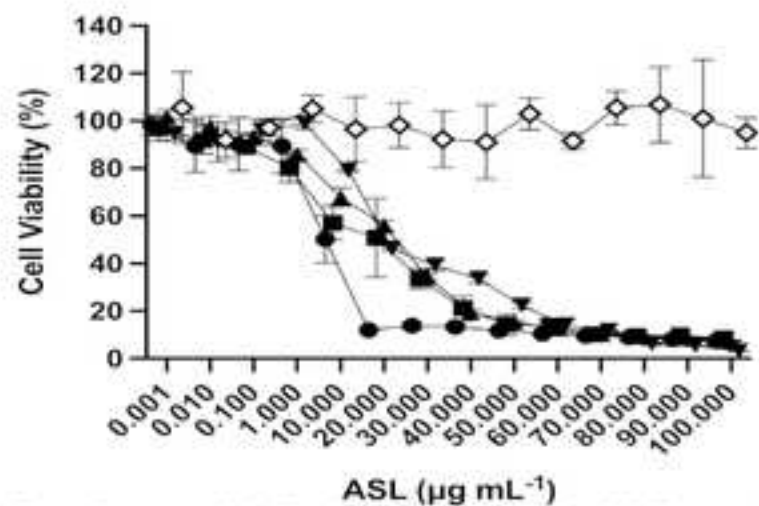
Acidic Sophorolipid



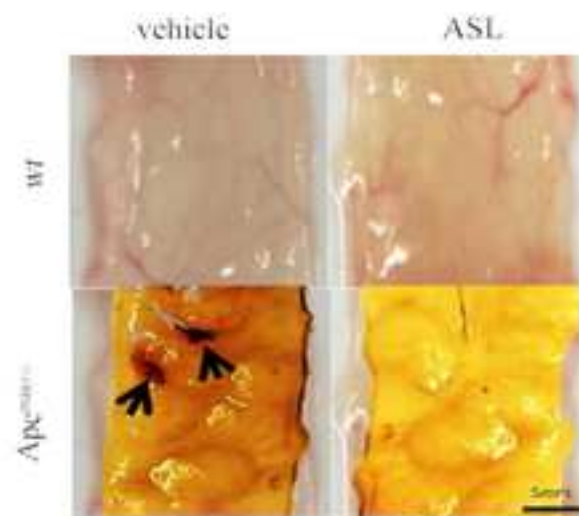
GI Cancer Cell Lines



APC^{min+/-} Mouse



Reduced Viability & Cell Death



Reduced Tumor Bleed



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