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Identification of rhein as the metabolite responsible for toxicity of rhubarb anthraquinones

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ABSTRACT: Rhubarb is a popular food in Europe with laxative properties attributed to anthraquinones. Long term usage of rhubarb anthraquinones has been linked to colonic toxicity, including the formation of melanosis coli, which is associated with increased risk of colon cancer. The major purgative anthraquinone in rhubarb is thought to be sennoside A, which is metabolised by colonic microflora. Here, we sought to identify the toxic metabolite responsible for melanosis coli in rats dosed with rhubarb anthraquinones for up to 90 days. Three metabolites were detected in rat faeces using HPLC. Of these, rhein was identified as the metabolite that accumulated most over time. Fecal flora from treated rats were capable of greater biotransformation of sennoside A to rhein compared to that from control rats. Cell culture experiments suggested that apoptosis and autophagy induced by rhein is the likely mechanism of chronic toxicity of rhubarb anthraquinones.

Keywords: Rhubarb anthraquinones, colonic toxicities, melanosis coli, rhein accumulation, apoptosis, autophagy

1. **Introduction**

Constipation is a common gastrointestinal disorder, which can result in infrequent stools, and difficult stool passage with pain and stiffness. The average global prevalence of constipation in adults has been estimated to be 16% of the population (Sanchez & Bercik, 2011). Traditional plant remedies such as *rhubarb, senna leaves, alder tree bark, cascara bark and aloe* contain anthraquinones as the active purgative ingredient, and consequently, anthraquinones are used in over the counter remedies for constipation (Camilleri et al., 2017). However, many of them have been historically linked with colonic toxicities of which the development of melanosis coli (MC) has attracted most attention (Willems et al., 2016; Chen et al., 2011).

MC is a benign colonic disorder but co-occurs with a variety of colon diseases and conditions including increased risk of colonic neoplasm (Biemacka-Wawrzonek et al., 2017; Coyne, 2013; Nusko et al., 1993). It is characterized by non-specific light to dark brown pigments in macrophages of the lamina propria and submucosa of the colon (Li et al., 2015). Its formation is thought to be caused by apoptotic cells which are ingested by macrophages and subsequently transported into the lamina propria, where lysosomes use them to produce lipofuscin pigment (Chen et al., 2011). Recently, the detection rate of MC has increased along with an increasing prevalence of constipation, aging of the population and advances in colonoscopic diagnosis (Wang et al., 2018; Liu et al., 2017). There is evidence of correlation between MC and the chronic use of anthraquinone-containing laxatives (Nesheiwat et al., 2020). After laxatives use is stopped, resolution of MC can take up to a year. Although the association of MC with the chronic use of anthraquinone-containing laxatives has been widely recognized, the toxic substances responsible are still not clarified. The main reason is that the laxative function of the anthraquinones is due to metabolites produced in the intestines.

Rhubarb is one of the most effective laxatives and is widely used in the treatment of intestinal constipation throughout the world. It is not only officially listed

in the Chinese Pharmacopoeia but also appears in the British Pharmacopoeia and European Pharmacopoeia (Sun & Yeh, 2005). As major laxative components, rhubarb anthraquinones (RA), belong to anthranoid glycosides and consist of a variety of anthraquinone derivatives including sennoside A, B, C and D, etc (Li et al., 2017). Sennoside A (SA) is thought to be the main laxative component and is widely used as a chemical marker for quality control for RA laxative products (Esposito et al., 2016). SA and anthraquinone derivatives are thought to be protected by a β-glucoside bond allowing them to reach the colon where they are degraded into complex metabolites by enzymes secreted from the intestinal microbiome (Matsumoto et al., 2012). It is these metabolites that actually produce the laxative functions through stimulating the intestinal wall innervation, modulating multiple aquaporin proteins, and increasing intestinal reflex peristalsis (Cao et al., 2018). Therefore, the cumulative toxicity of the metabolites might be unavoidable when the RA products are used chronically. We hypothesized that some metabolites might accumulate in the colon and contribute to the toxicities including MC formation.

In this study, rats were fed with the RA for 90 days and were investigated for diarrhea grade and pathological changes, with fecal samples obtained regularly. An HPLC method was used to analyze RA metabolites remaining in the rat feces. *In vitro* biotransformation was generated by using SA cultured in fecal flora isolated from fresh feces of the RA treated rats (RA-rats) and normal control rats (NC-rats). Furthermore, the induction of apoptosis and autophagy was investigated in normal colon cells as possible mechanisms of toxicity.

2. Materials and Methods

2.1. Chemicals, Reagents and Rhubarb Anthraquinones (RA)

Single compounds (sennoside A, rhein, emodin and aloe-emodin) were supplied by Sichuan Victory Biological Technology Co., Ltd. (Chengdu, China).

Methanol used for HPLC analysis was of chromatographic grade and purchased from Comio Chemical Reagent Co., Ltd. (Tianjin, China). All organic solvents applied for extraction and separation were of analytical grade and purchased from Tianli Chemical Reagent Co., Ltd. (Tianjin, China). Ultrapure water was produced by a reverse osmosis Milli-Q (18MΩ) system (Fuller Technology Co., Ltd, Qindao, China).

Dried roots of Rhubarb (*Rheum palmatum L.*) were purchased from commercial sources: Shaanxi Pharmaceutical Holding Group Co., Ltd. (Xi'an, China). The roots were extracted with 50% ethanol and the solvent was evaporated under vacuum to obtain a crude extract. The crude extract was dispersed in water and poured onto a D101 Macroporous adsorption resin column (Lanxiao Technology New Materials Co., Ltd., Xi'an, China), and eluted with water (3-fold of column volume) followed by 60% ethanol (6-fold of column volume). The RA in 60% ethanol eluent was evaporated to dryness under vacuum, yielding 9.65 g of RA per 100 g of the dried rhubarb. The content of SA was 4.56% (w/w) based on HPLC analysis. The RA was stored at -20 ℃ for later experiments.

2.2. Experimental Animals

Pathogen free male Sprague-Dawley (SD) rats were purchased from Dashuo experimental animal Co. Ltd (Chengdu, China) with the license number (certificate No. SCXK (chuan) 2014-028). Prior to the experiment, all animals were acclimatized in a pathogen-free-grade animal room under controlled conditions (24±1.0 °C, 60±5% humidity with a 12h/12h light-dark cycle) for seven days and received standard laboratory chow and tap water *ad libitum*.

All procedures for the care and handling of animals used in the study were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986 (Amended 2012) and associated guidelines, EU Directive 2010/63/EU for animal experiments, following ethical review by the Animal Care Committee of Northwest University and were approved by the Animal Experimental Center of Northwest University.

2.3. Animal Experiments and Sample Collections

The experiment lasted for a total of 90 days. The rats were randomly assigned to either normal control group (NC-rats) or RA-treated group (RA-rats), with 8 rats in each group. They were dosed daily with distilled water (NC-rats) or the RA solution by oral gavage (RA-rats) for 90 days. During the dosing period, all the rats were given free access to food and water. Body weight, diarrhea grade and behavior of rats was recorded.

The fresh fecal samples were collected from the rats every 6 days, 3 hours after dosing, and processed immediately to prepare fecal flora solution (FFS) using a slight modification of a published method (Song et al., 2011). Samples from all eight rats in control or treatment groups were pooled. Briefly, the fresh feces were added to sterile physiological saline at a ratio of 1:5 (w/v), and mixed thoroughly under anaerobic conditions. The mixture was then centrifuged at 3000 rpm for 10 min at 4 ℃. The supernatant, FFS, was divided into two portions. One portion was used for HPLC analysis (described in 2.5), and the other portion was used for the biotransformation study (described in 2.6).

On the last day of the experiments, rats were euthanized by intraperitoneal injection of 10% urethane solution (0.10 ml/kg body weight) 3 hours after the last treatment, and organs including colon, stomach, lung, kidney, heart, liver, spleen,

testis, prostate, thymus and adrenal gland were collected. These were evaluated for organ index, and the colons were fixed in 4% paraformaldehyde and embedded in paraffin for hematoxylin and eosin (H&E) staining.

2.4. H&E Staining on the Colon Tissues

The colon tissues embedded in paraffin were sectioned into 0.5 μm slices that were placed on glass slides. The slides were deparaffinized and stained with H&E followed by being dehydrated in alcohol, cleared in xylene, and covered for imaging under a light microscope. Assessment was made of the destruction of the crypt structure, the depth of the lesions, the degree of inflammatory cell infiltration and brown staining. MC formation was judged by the colour of the colonic membrane.

2.5. Analysis of RA metabolites in fecal samples by HPLC

One portion of the FFS (described in 2.3) was added with an equal volume of methanol followed by centrifugation at 12000 rpm for 10 min at 4 ^{o}C. The supernatant was filtered (0.22 μm pore size) and stored at -20 ^{o}C for later HPLC analysis.

The HPLC system was equipped with a Shimadzu DGU-20A_3 online degasser, two Shimadzu LC-20AD pumps and SPD-M20A PDA detector (Shimadzu, Japan). The chromatographic separation of the analyte was achieved by a C18 column (4.6×250 mm, 5 μm, Hanbang, China) at 35 ^{o}C, with a mobile phase consisting of methanol (A) and water containing 0.1% of phosphoric acid (B) at a flow rate of 1 ml/min. The gradient elution was performed as follows: 10%～27% A at 0～5 min; hold 27% A at 5～10 min; 27%～53% A at 10～25 min; 53%～80% A at 25～40 min and 80%～80% A at 40～55 min. The injection volume was 10 μl and the detection wavelength was set at 280 nm. Standard solutions of the single compounds were used for identification and quantification of the metabolites produced.

163 *2.6. Biotransformation of SA by Fecal Flora*

164 The ability of the FFS to metabolise SA was determined with one portion of the
165 FFS (described in 2.3) added to a solution of 0.375 mM SA. The mixture was
166 incubated at 37 ^{o}C under anaerobic conditions and the reaction was terminated at
167 designated times (0 and 4 h) by adding methanol with equal volumes of the cultured
168 solution. After centrifugation at 12000 rpm for 10 min, the supernatant was filtered
169 (0.22 μm pore size) and stored at -20 ^{o}C until HPLC was performed.

170 *2.7. Cell Lines and Cell Culture*

171 The human colorectal carcinoma cells, HT29 (ATCC, Manassas, USA), were
172 cultured in DME/F-12 medium (HyClone, China), supplemented with 10% (v/v) fetal
173 bovine serum (Royacel, China). The human normal colonic epithelial cell line,
174 NCM460 cells (INCELL, San Antonio, TX, USA), were grown in RPMI 1640
175 medium (Corning, USA) supplemented with 10% (v/v) fetal bovine serum (Royacel,
176 China) in a humidified atmosphere containing 5% carbon dioxide at 37 ^{o}C.
177 Exponentially growing cells were used in all experiments. All cell lines were negative
178 for *Mycoplasma* contamination.

179 *2.8. Cell Viability Analysis*

180 Cells were seeded in triplicate into 96-well plates at a density of 4×10^4 cells/ml.
181 Twenty-four hours after seeding, the cells were cultured in medium containing various
182 concentrations of the drugs. At each designated time, cell viability was determined
183 using the sulphorodamine B (SRB) assay. Briefly, cells were fixed in 10%
184 tri-chloroacetate (Shanghai Shanpu chemical Company Ltd., China) for at least 1 hour
185 at 4 ^{o}C, washed with tap water and allowed to dry before being stained with 0.4%
186 SRB (Sigma, China). The bound SRB was dissolved in 10 mM Tris and absorbance
187 read at 490 nm by a microplate reader (Synergy2, BioTek Instruments Ltd., USA).

2.9. Acridine Orange Staining

Acridine orange staining was performed to visualize autophagic vesicles and nuclear DNA. The cells were seeded at a density of 1×10^5 cells/ml on slides for 24 hours, and treated with rhein at a concentration of 10 μM or 40 μM. After 3 or 24 hours, the cells were washed with PBS and stained by using 27 μM of acridine orange for 10 min in dark at a temperature of 37 ^{o}C. Subsequently, the cells were thoroughly washed and imaged with blue and green fluorescence under a fluorescence microscope (Nikon Eclipse TE2000-U, Japan).

2.10. Data Analysis

Statistical analyses were performed using SPSS statistic 20 software and GraphPad Prism version 5.0 (GraphPad Software, Inc., CA, USA). All results are expressed as the mean ± standard error. Statistical comparisons between groups were made by Student's *t* test. Results with $p<0.05$ was considered to be statistically significant.

3. Results

3.1. Purgative Action and Drug Tolerance Shown in the Rats Administered RA

The diarrhea grade was assessed based on the fecal stool consistency, which was set as normal (separate solid lumps), loose stool (mushy stool with a few solid pieces) or watery diarrhea (watery without solid pieces). To properly evaluate the diarrhea grade, we measured water content in feces by weighing before and after drying at 60℃ for 24 h. The normal control rats (NC-rats) showed a normal stool with the mean water content of 63.14±4.71% ranging from 56.8 % to 71.01 % (Supplementary Figure A). The initial RA dose of 48.25 mg/kg was based on preliminary experiments (data not shown) in which this dose was found to induce loose stools in the rats. At

213 this initial dose, the rats had loose stools with a water content of 79.56 %. Following
214 daily treatment, the rats showed drug resistance with the disappearance of the loose
215 stools in 80 % of the rats. The RA dose had to be gradually increased in order to
216 maintain the purgative action. Therefore, the fecal water content in the RA-rats
217 increased at each new dosage of the RA used, and diarrhea was observed. Following
218 the treatment for a few days, the water content in the feces decreased and the normal
219 stools were observed. During the consecutive 90 days, the RA dose was gradually
220 increased up to 10 times from 48.25 mg/kg to 482.5 mg/kg per day (Fig. 1A). The
221 diarrhea grade in the RA-rats, however, gradually declined to normal levels. For
222 example, the water content in the RA-rats decreased down to 65.60% at day 60. The
223 feces collected from the RA-rats had a wide range of water content from 65.60% to
224 92.91% (Fig. 1A). It was not possible to accurately predict how well an increased
225 dose would restore the laxative function, which is why there is a sharp increase in
226 water content at around 60 days, although it was not possible to increase this back
227 above normal.

228 During the 90 days, the treatment resulted in significant reduction in the rats'
229 body weights. The NC-rats exhibited normal growth throughout the study with an
230 increase in body weight of 18.62 %, whereas the RA-rats showed a decline in body
231 weight with a reduction of 3.38 % of starting weight at the end of the last day (Fig.
232 1B).

233

234 Figure 1 here

235 *3.2. Pathological Changes in Colons of the Rats Administered with RA for 90 Days*

236 On the last day of the experiments, all the rats were sacrificed and the following
237 organs were collected: colon, stomach, lung, kidney, heart, liver, spleen, testis,
238 prostate, thymus and adrenal gland. They were weighed and analyzed with the organ

239 index (organ body weight ratio) calculated (Fig. 2A). Compared to the NC-rats, the
240 RA-rats showed significant increases of the colon (p=0.0001) and stomach index
241 (p=0.0044), and had significant decreases of the testis index (p=0.0364). Our previous
242 study demonstrated that oral administration with anthraquinones could result in
243 change of expression of aquaporins (AQPs) in the rat tissues (Cao et al., 2018). AQPs
244 are known to modulate water transport in intracellular and extracellular cells, so these
245 changes in gene expression of AQPs may change water content of the tissues. This
246 could explain the increase on the stomach index and colon index. This will be
247 investigated further in our future studies. In addition, there was no statistically
248 significant difference for other organ index results between the two groups.
249
250

Fig.2 here

251 To look at pathological changes in the colon, we performed histopathological
252 examination. The H&E staining revealed severe damage in the colons of the RA-rats.
253 In the NC-rats (Fig. 2B), the colons displayed intact epithelium and mucosa, no
254 disruption of crypt architecture, complete goblet cells with mucus-filled vacuoles, and
255 no infiltration of leukocytes. There was no pigmentation shown in the NC-rats with
256 the overall pink color in the whole colon tissues.
257 However, severe damage with decreased goblet cells was found in the colons
258 collected from the RA-rats (Fig. 2C). The colonic section of the RA-rats showed
259 decreased goblet cells and inflammatory cell infiltration in the crypt architecture. In
260 particular, MC formation was seen, with a diffuse and deep yellow-brown color on the
261 colonic membrane, and with brown pigments distributed in the lamina propria.

262 *3.3. Rhein Accumulation Shown in Feces of the RA-treated Rats*

263 To detect the RA metabolites remaining in the feces, the fecal samples collected
264 from the RA-rats were pooled and analyzed by HPLC. Under the HPLC conditions
265 applied, most of the RA components were clearly separated and eluted before 40.0
266 min (Fig. 3A). The component SA was identified with a retention time at 30.4 min,
267 which was confirmed by use of a standard chemical (Supplementary Figure B). In the
268 feces collected from the RA-rats, peaks of the RA components decreased significantly
269 or even completely disappeared (Fig. 3B). Three additional peaks were present at 42.6
270 min (M2), 45.6 min (M3) and 47.7 min (M4) that were not found in the HPLC
271 profiles of the original RA or in feces collected from the NC-rats (Fig. 3C). Therefore,
272 it was concluded that M2, M3 and M4 were RA metabolites present in the colon. By
273 comparison to standard chemicals, M2 and M3 were identified as aloe-emodin and
274 rhein, respectively (Supplementary Figure C and D).
275
276
Fig. 3 here
277 To investigate the possible accumulation of the RA metabolites, we collected
278 feces regularly from the RA-rats during the 90 days of the experiment. A total of
279 sixteen fecal samples were obtained and the metabolite profiles analysed using the
280 HPLC method (Fig. 3D). It can be seen that SA and M4 were present at very low
281 levels and M2 was only detected occasionally. M3 (rhein) was present at variable
282 levels in samples from different time points but generally increased over time. Overall,
283 the average content followed the order M3 (rhein)>SA>M4>M2 (Fig. 3E). Based on
284 the peak area, the rhein concentration reached the highest (66.62 μg/g) in the feces
285 collected at day 80.

286 *3.4. Biotransformation of SA with fecal flora of the RA-rats*

To show that fecal flora could transform SA, we treated SA *in vitro* with fresh FFS obtained from both NC-rats (collected on day 0) and RA-rats (collected on day 23). FFS from both NC and RA-rats was able to biotransform SA into five products seen on HPLC chromatograms (Fig 4A and 4B), with more metabolism apparent in the SA treated with FFS from RA-rats. HPLC analysis of the reaction products shows one SA peak at 30.4 min and five additional peaks at 38.9 min (M1), 42.6 min (M2), 45.6 min (M3), 47.7 min (M4) and 50.8 min (M5) (Fig. 4A and 4B). By using standard chemicals, M2 was shown to be aloe-emodin (Supplementary Figure C); M3 was identified as rhein (Supplementary Figure D). M1, M4 and M5 were not identified and will be investigated in the future.

The experiment was repeated with pooled samples (n = 8) from each of 16 sampling points over the 90 day experiment. Fig 4 C-F shows the fold variation compared to day 0, in four of the five metabolites when SA was treated with FFS from the 16 different samples. M2 was not measurable in most of the samples and is not included. It can be seen that the ability of FFS from RA-rats to produce rhein increased over time (Fig. 4E) suggesting that bacteria responsible for this metabolism may have increased over time in the GI tract of the treated rats.

Fig. 4 here

Overall, the peak area of the metabolites followed the order M4 > M5 > M3 (rhein) > M1 (Fig. 4G). A significant increase in rhein production was seen in the RA-FFS ($p<0.0001$) compared to the NC-FFS. No significant changes on production of M1, M4 and M5 were observed.

3.5. Cell Growth Inhibited by Rhein

In order to assess the potential cytotoxic effect of SA and rhein, SRB assays were

carried out to determine viability of the normal colon cells (NCM460) and colon cancer cells (HT29) treated with or without the drugs for five days. We found that the SA did not influence the growth of either cell line at doses ranging from 5-100 μM (Fig. 5A).

Fig. 5 here

However, the rhein showed a dose-dependent inhibition of growth in both cell lines (Fig. 5B). The inhibition was greater in HT29 cells than in NCM460 cells. The EC_{50} of the rhein was around 20 μM in HT29 cells, and was around 50 μM in NCM460 cells. The treatment with rhein at 40 μM resulted in 77.76% viable cells for NCM460 and 27.70% viable cells for HT29.

3.6. Autophagy and Apoptosis Induced by the Rhein Treatment

To observe the induction of apoptosis, NCM460 cells were treated with rhein at 40 μM for 24 hours, and stained with acridine orange. The untreated NCM460 cells had a normal shape and uniformly stained nucleus. In the treated NCM460 cells, in contrast, massive cells were observed with nuclear DNA breakage indicating the apoptotic status of the cells (Fig. 6A).

To look at induction of autophagy, NCM460 cells were treated with or without rhein at 10 μM. After three hours, red fluorescence in the treated cells was enhanced, with extensive punctuated distribution indicating the presence of autophagic vacuoles in the treated NCM460 cells (Fig. 6B). Thus, the rhein treatment could induce autophagy as well as apoptosis in the normal colon cells.

Fig 6 here

4. Discussion and conclusion

With the increased prevalence of constipation, anthraquinone-laxatives are regularly taken by many people around the world (Malik & Müller, 2016; Kunkel et al, 2009). Surveys indicate that drug-tolerance is one of the initial problems associated with long-term usage of anthraquinone-laxatives (Yokoyama et al, 2017). Over time, patients have to be given higher doses to maintain normal defecation, with symptoms of ongoing constipation and weight loss occurring in these patients (Cirillo & Capasso, 2015). In our study, the rat model mirrored what has been observed in patients, with the need to gradually increase the RA dose, as the purgative effect decreased or completely disappeared during the 90 days. Treated rats also showed weight loss, and inflammation and pigment deposition in the colon tissues.

RA, as the main effective components of the laxatives, undergo metabolism in the colon. In general, most of the RA metabolites are absorbed into the blood stream within 2 hours and removed through other organs within 24 hours after oral administration (Huang et al, 2019). However, rhein has a longer half-life and a lower clearance rate. After single intracaecal administration of [^{14}C] rhein (25 mg/kg) to rats, the recovery rate of ^{14}C after five days was 37% in urine and 53% in faeces (De Witte & Lemli, 1988). It is no surprise, therefore, that in our experiments the rhein was found to accumulate in colons of rats during long-term treatment with the RA. Furthermore we were able to show that fresh FFS can transform SA to produce the same metabolites observed from the *in vivo* study, including rhein. This biotransformation was higher with FFS from RA treated rats, suggesting that the microbial enzymes responsible may be induced by exposure to RA.

Rhein, also known as 1,8-dihydroxy anthraquinone-3-carboxylic acid (Supplementary Figure E), has been revealed to have pharmacological effects

including hepatoprotective, nephroprotective, anti-inflammatory, antioxidant, anticancer and antimicrobial activities (Zhou et al, 2015). Its anticancer activity has been shown to be due to mechanisms including apoptotic and autophagic induction in various cancer cells (Sun et al, 2016). Our study showed that rhein induced more potent inhibition on colon cancer cells than on the normal cells. This property might allow rhein to be developed as an anti-cancer drug, although there may be some toxicity to the normal colon cells.

It is well known that apoptosis and autophagy are the main mechanisms involved in programmed cell death. Apoptosis is characterized by cell changes including cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Autophagy is an intracellular degradative system that is characterized by the formation of lysosome vesicles. Using acridine orange staining, we found that the rhein treatment caused extensive autophagic vacuoles at low dose and resulted in nuclear breakage at high dose to the normal colon cells. It has been proposed that MC is due to apoptotic and autophagic cells causing pigment formation in the colon only (Chen et al., 2011). Our study shows that rhein formed in the colon from microbial biotransformation of SA is the most likely cause of the toxicity that presents as MC.

Interestingly, a clinical finding of MC diagnosis due to the usage of diacerein was reported recently (Jiménez et al., 2017). Diacerein, a drug used for osteoarthritis treatment, is a prodrug of rhein. It has been shown that diacerein could be entirely converted into rhein before reaching the systemic circulation. However, the apparent bioavailability of rhein ranges between 35-56%. The incomplete absorption of rhein from the upper part of the gastrointestinal tract means that rhein could be transported to the colon leading to some side effects (Mandawgade et al., 2016). This MC diagnosis is clinical evidence to support our findings.

In summary, we showed that the long-term usage of RA could result in drug-tolerance, inflammatory changes in colonic architecture, MC formation and rhein accumulation in the colon. Rhein accumulation could induce cell apoptosis and autophagy, which might contribute to colonic toxicities including MC formation. Therefore, strategies aimed to mediate rhein accumulation in the colon might help to reduce toxicity to the colon.

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Conflicts of interest

The authors declare no conflicts of interest.

Abbreviations

FFS, fecal flora solution; H&E, hematoxylin and eosin; HPLC, high-performance liquid chromatography; MC, Melanosis coli; NC, normal control; RA, rhubarb anthraquinones; SA, sennoside A; SD, Sprague-Dawley; SRB, sulphorodamine B.

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483

484 **Figure Legends**

Fig. 1. The Sprague-Dawley rats were fed with the rhubarb anthraquinones (RA) for 90 days. (A) The RA dose given to the rats and the water content in the feces of the RA-rats (n=8). (B) The transition of body weight in the normal control rats (NC) and the RA-rats (RA). Data is represented as mean ± SD (n=8).

485

Fig. 2. Pathological changes in the rats fed with the rhubarb anthraquinones (RA) for 90 days. (A) Organ index (organ body weight ratio) shown in the normal control rats (NC) and the RA-rats (RA). Data is reported as mean ± SD (n=8). * represents significant difference with the $p<0.05$, ** represents significant difference with the $p<0.001$, *** represents significant difference with the $p<0.0001$. (B) The H&E staining on the colons collected from the NC-rat (10× and 40×). (C) collected from the RA-rat (10× and 40×).

Fig. 3. Rhubarb anthraquinones (RA) metabolites in the rat feces. (A) HPLC chromatogram of the RA; (B) of the feces collected from the RA-rats (n=8); (C) of the feces collected from the NC-rats (n=8). (D) content of sennoside A (SA) and metabolites (M2, M3 and M4) in the feces of the RA-rats (n=8) at different days; (E) content in the feces during the consecutive 90 days.

486

Fig. 4. Biotransformation of sennoside A (SA) was achieved by being cultured at 37℃ in the rat fecal flora solution (FFS) for 4 hours. (A) HPLC chromatogram of the SA degraded in the FFS obtained from normal control rats (NC, n=8) at day 0; (B) from RA-rats (RA, n=8) at day 23. (C) content of M1 produced; (D) content of M3 (rhein) produced; (E) content of M4 produced; (F) content of M5 produced. (G) average content of each components in the FFS during 90 days.

Fig. 5. The normal colon cells (NCM460) and colon cancer cells (HT29) were cultured in the medium with or without sennoside A (SA) or rhein for 5 days. The cell viability was determined by using sulphorodamine B (SRB) assays. Data are the mean ± SD of three independent experiments, relative to the untreated cells. (A) The cells were treated with the SA for five days. (B) The cells were treated with the rhein for five days.

Fig. 6. The normal colon cells (NCM460) were treated with rhein, followed by staining with acridine

orange. The stained cells were imaged (x20 times) with green and red fluorescence under a fluorescence microscope. (A) NCM460 cells were treated with or without 40 μM rhein for 24 hours. The DNA breakage (indicated by red arrows) was shown in the treated cells. (B) NCM460 cells were treated with or without 10 μM rhein for 3 hours. The punctured autophagic vacuoles (indicated by white arrows) was observed to be frequent in the treated cells.

487

Figure 1

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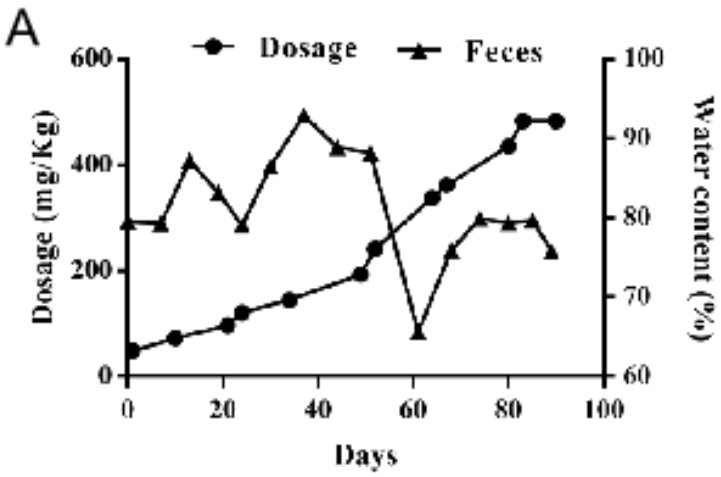

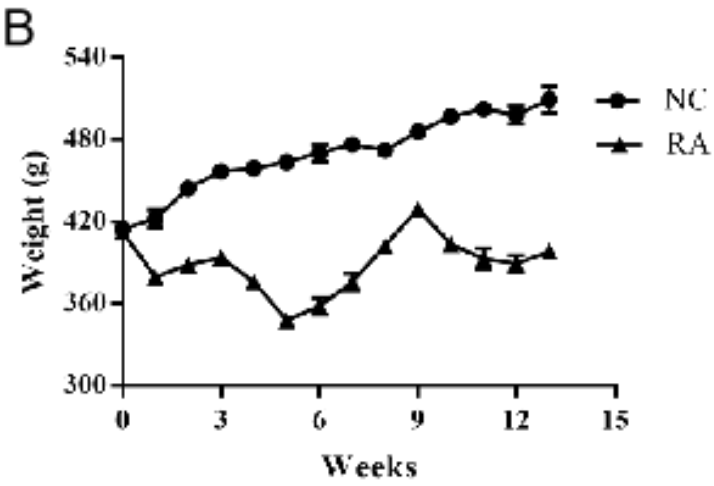

Figure 2

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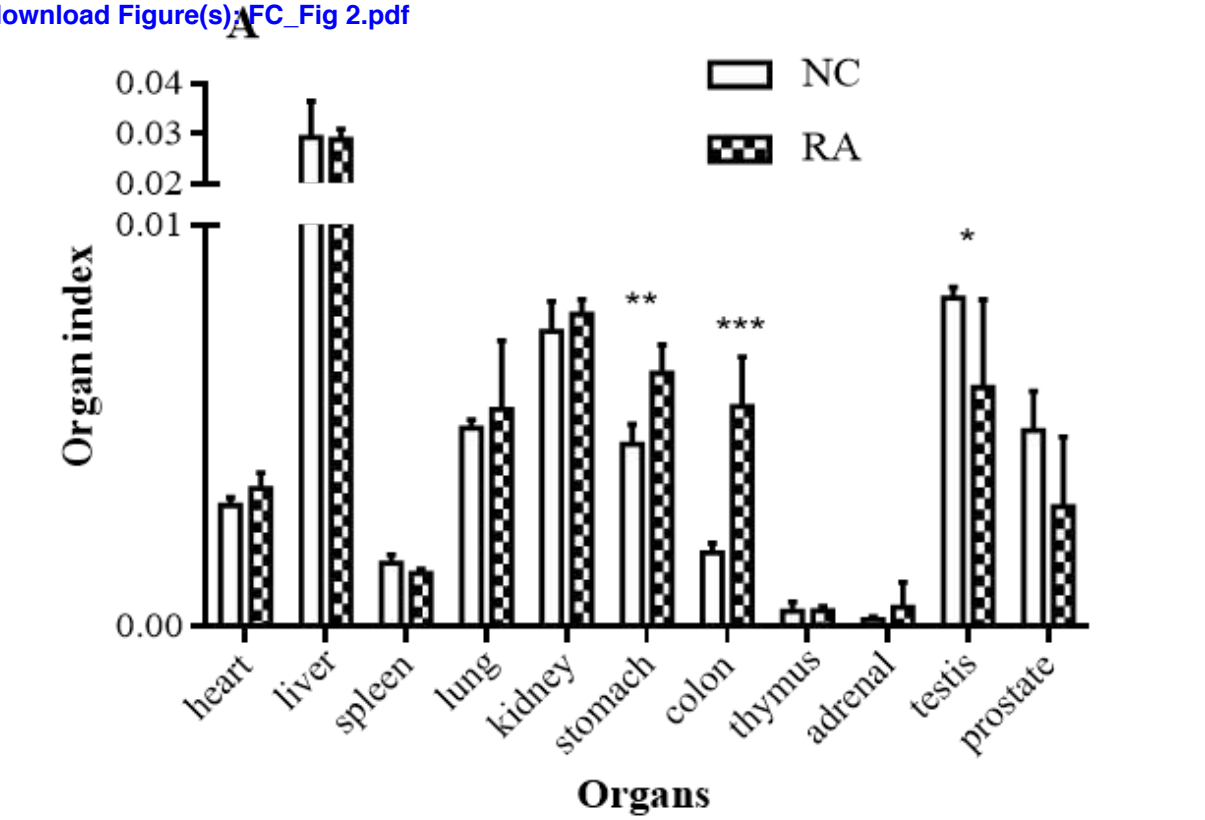

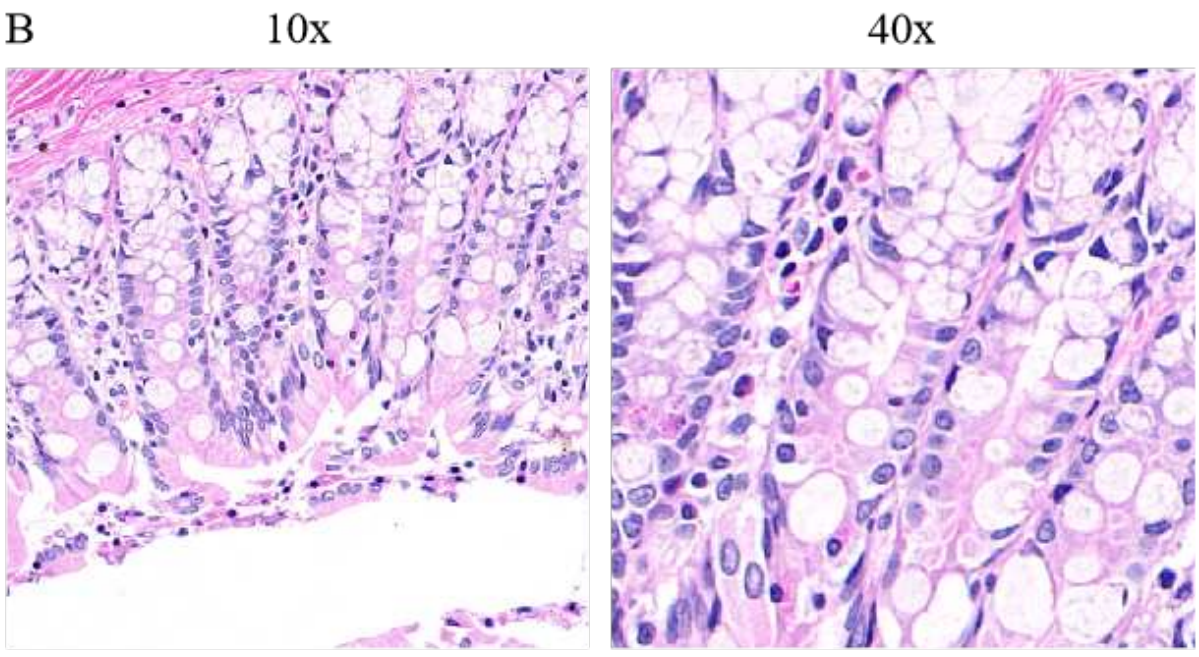

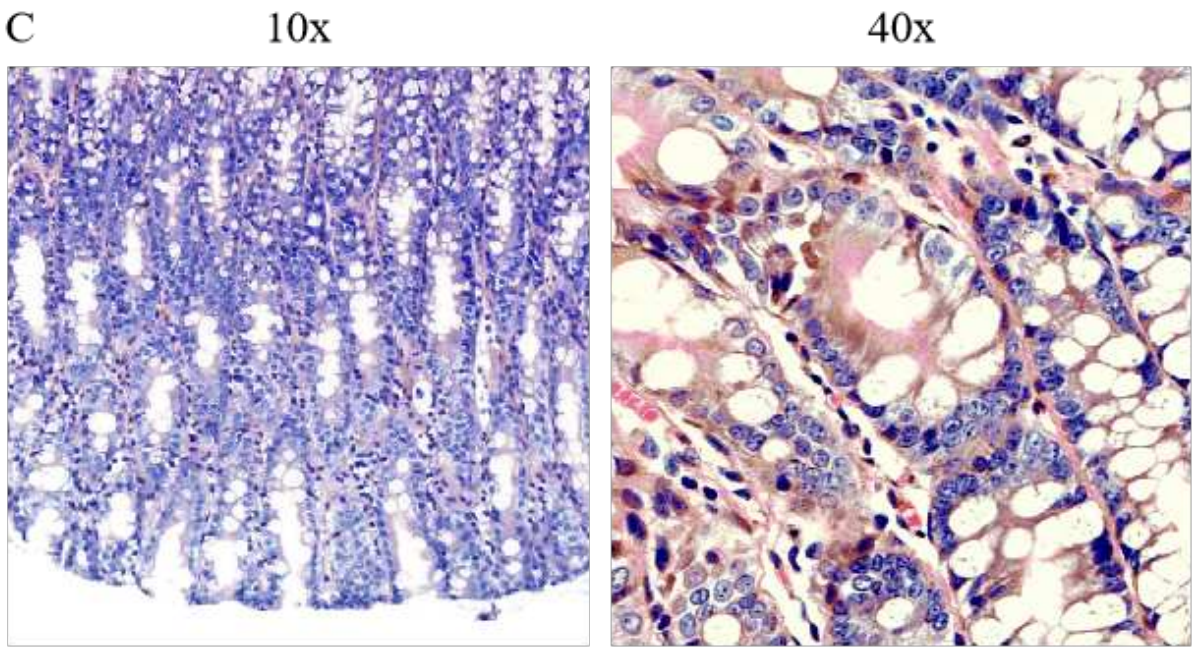

Figure 3

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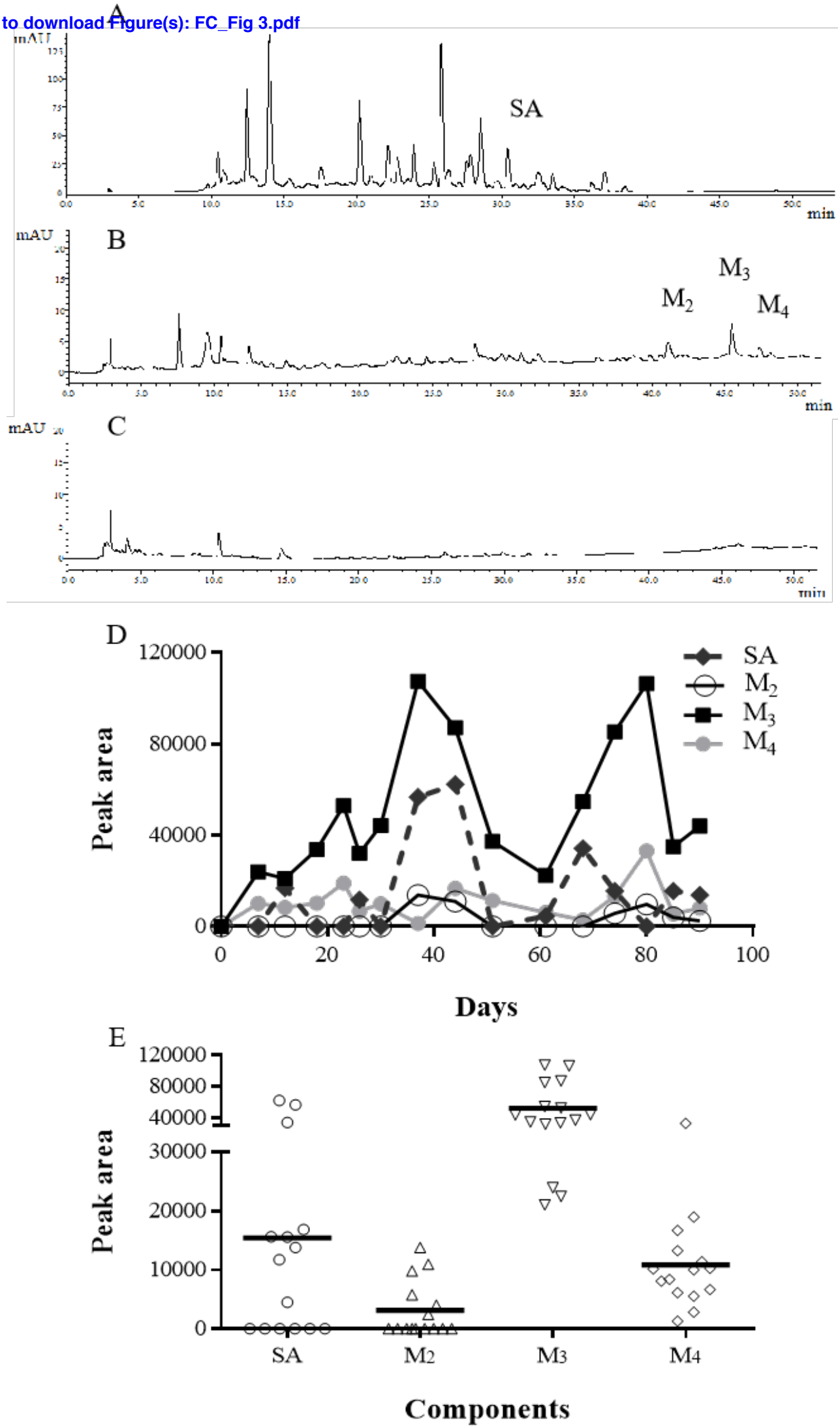

Figure 4

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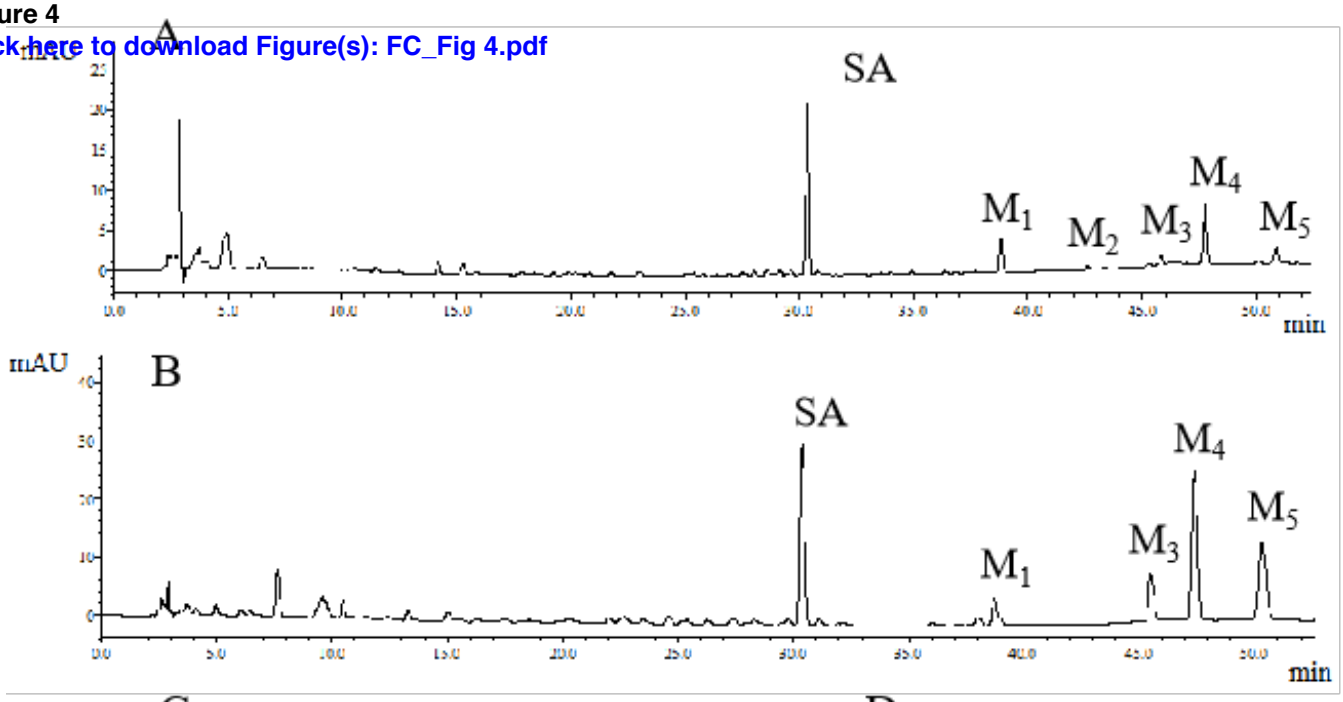

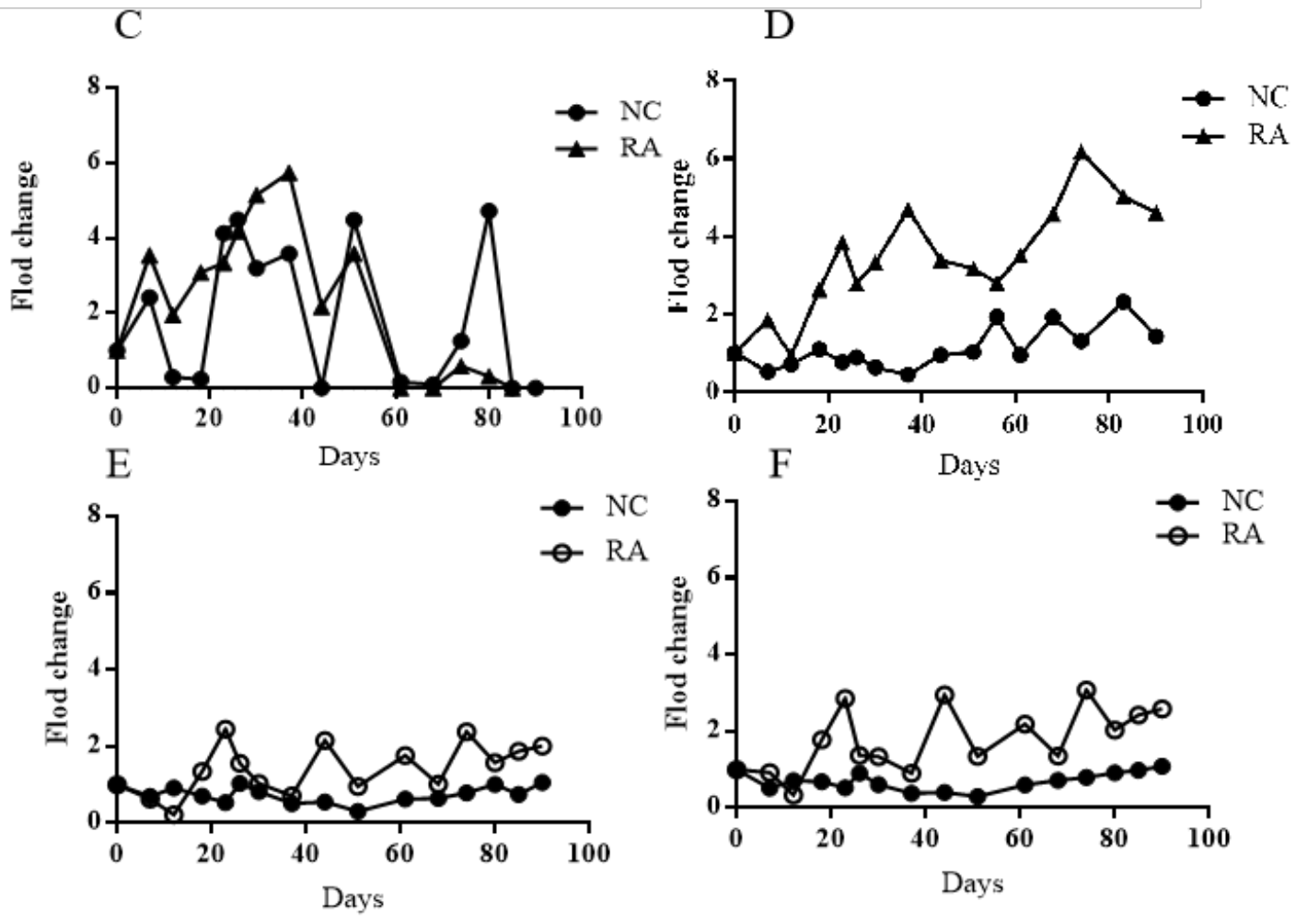

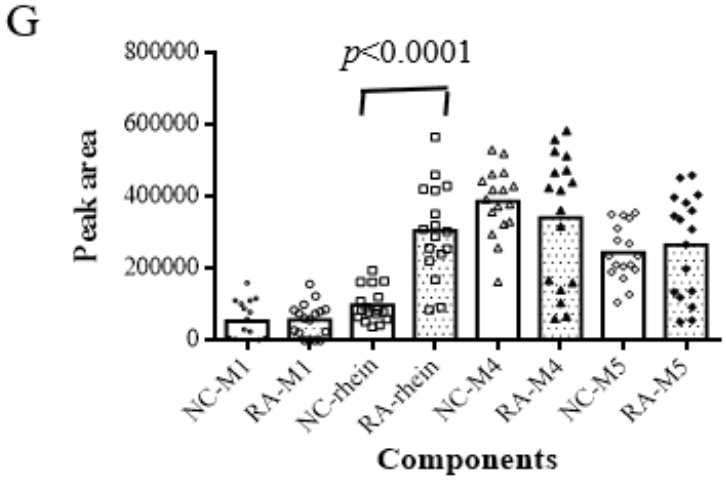

Figure 5

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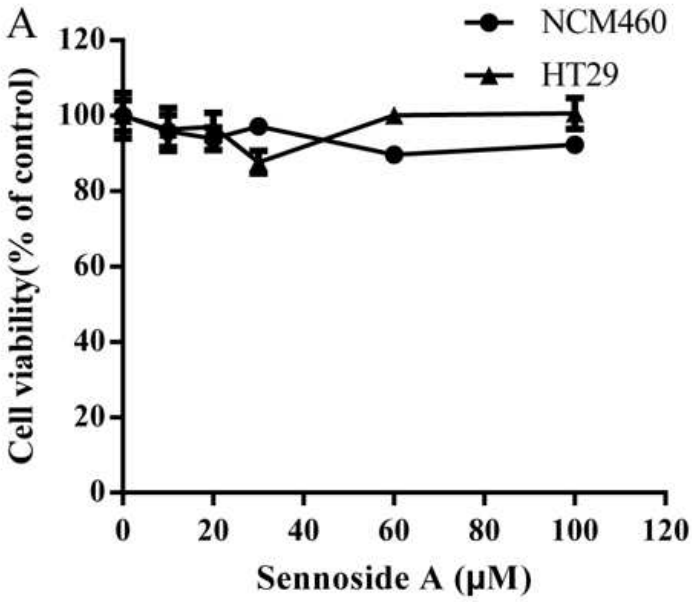

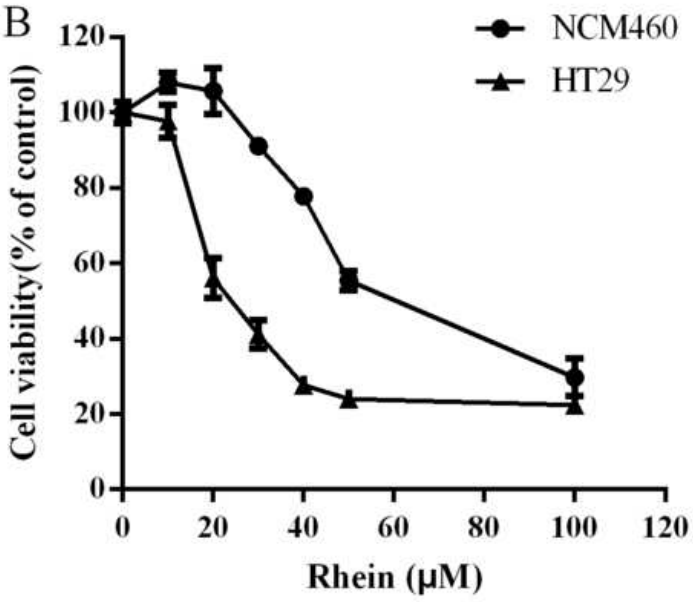

Figure 6

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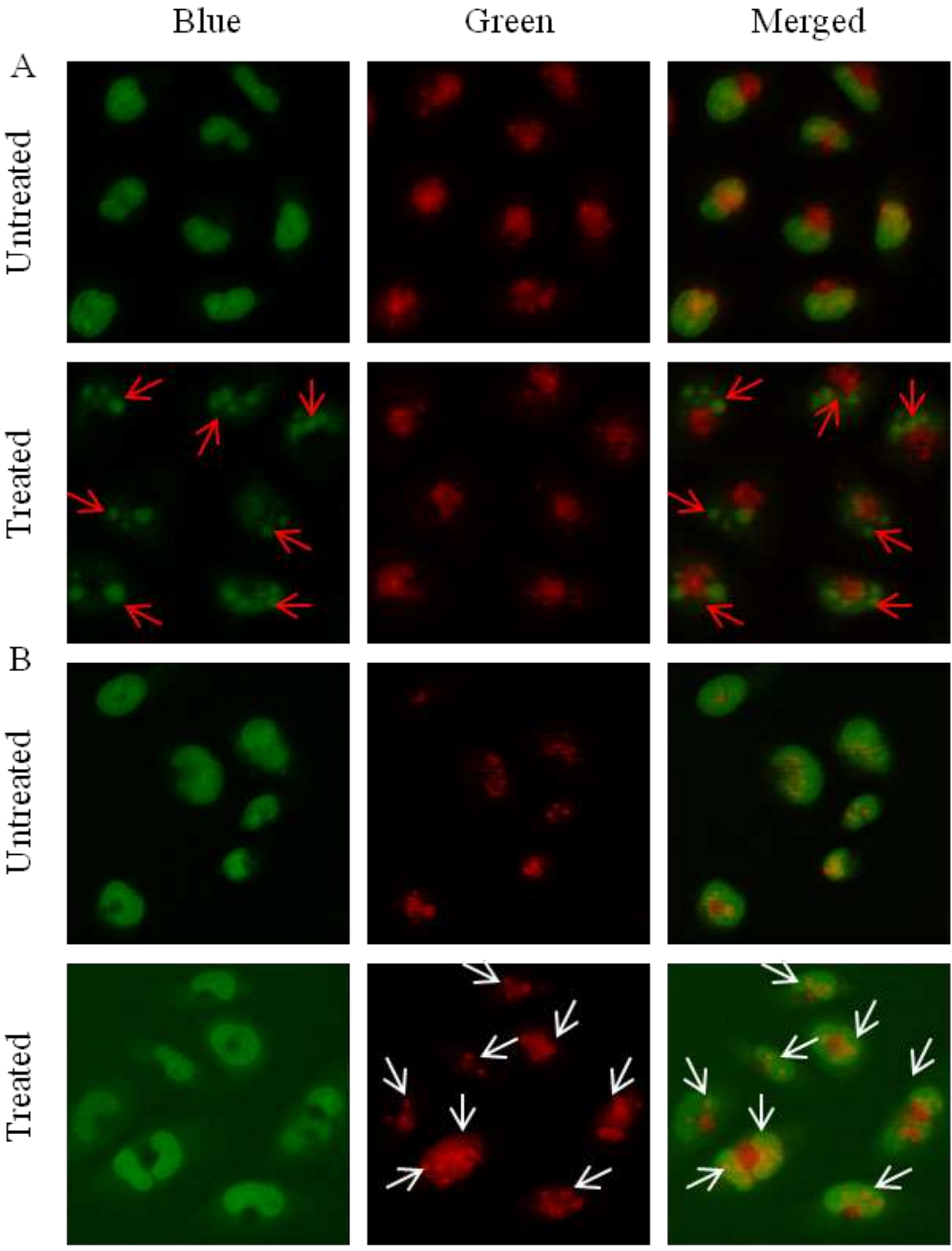
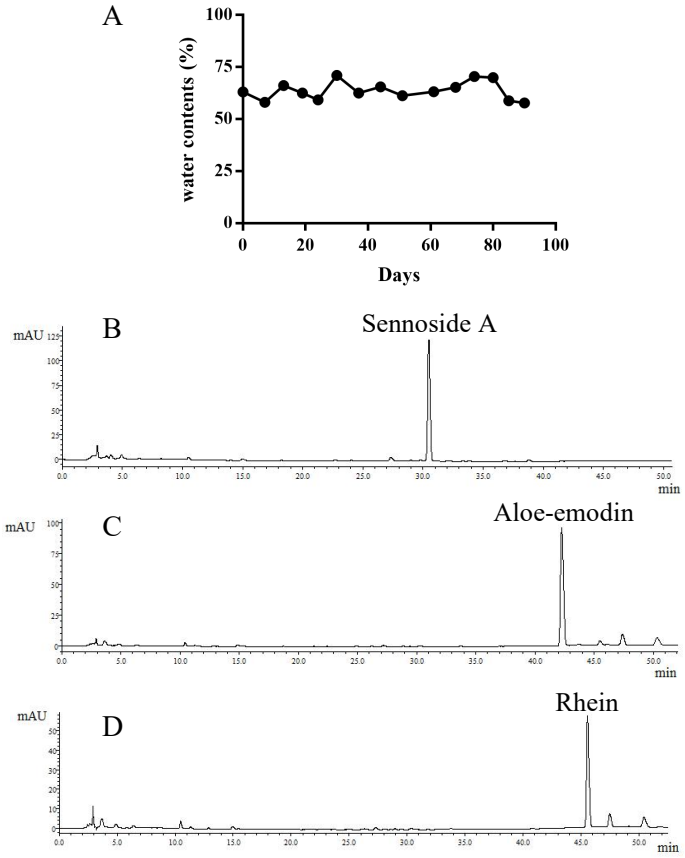
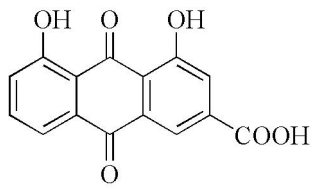


Appendix A Supplementary Data



Sennoside A

E



Supplementary Figure: (A) Water content in feces of the normal control rats. (B) HPLC chromatogram of sennoside A. (C) of aloe-emodin. (D) of Rhein. (E) chemical structure of rhein.