

Protective effects of Ursolic acid and Luteolin against oxidative DNA damage include enhancement of DNA repair in Caco-2 cells.

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27 **Abstract**

28 Consumption of fruits and vegetables is associated with a reduced risk of developing a  
29 wide range of cancers including colon cancer. In this study, we evaluated the effects of  
30 two compounds present in fruits and vegetables, ursolic acid, a triterpenoid, and  
31 luteolin, a flavonoid, on DNA protection and DNA repair in Caco-2 cells using the  
32 comet assay.

33 Ursolic acid and luteolin showed a protective effect against H<sub>2</sub>O<sub>2</sub>-induced DNA  
34 damage. To evaluate effects on induction of base oxidation, we exposed cells to the  
35 photosensitiser Ro 19-8022 plus visible light to induce 8-oxoguanine. Luteolin  
36 protected against this damage in Caco-2 cells after a short period of incubation. Repair  
37 rate was increased by pre-treatment of cells for 24h with ursolic acid or luteolin  
38 (rejoining of strand breaks) in Caco-2 cells after treatment with H<sub>2</sub>O<sub>2</sub>. We also  
39 measured the incision activity of a cell extract from Caco-2 cells treated for 24h with  
40 test compounds on a DNA substrate containing specific damage (8-oxoGua), to evaluate  
41 effects on base excision repair activity. Preincubation for 24h with ursolic acid  
42 enhanced incision activity in Caco-2 cells. In conclusion, we demonstrated for the first  
43 time that ursolic acid and luteolin not only protect DNA from oxidative damage but also  
44 increase repair activity in Caco-2 cells. These effects of ursolic acid and luteolin may  
45 contribute to their anti-carcinogenic effects.

46

47

48 **Keywords:** ursolic acid; luteolin; DNA oxidation; antioxidants; DNA repair; comet  
49 assay.

50

## 51 **1. Introduction**

52 Colorectal cancer (CRC) is one of the main causes of cancer-related mortality in the  
53 western world and was the second most common cancer in Europe in 2006 [1].

54 Oxidative stress, defined as a disturbance in the equilibrium status of pro-oxidant and  
55 antioxidant systems in favour of pro-oxidant, can damage diverse cellular  
56 macromolecules such as DNA, lipids, and proteins. The various types of DNA damage  
57 that can be generated as a result of oxidative attack, if not properly removed, can lead to  
58 mutagenesis and/or cell death. 8-oxo-7,8-dihydroguanine (8-oxoGua) is one of the most  
59 abundant forms of DNA oxidation and can cause G to T transversions in several  
60 oncogenes and tumour suppressor genes [2]. The major mechanism repairing DNA  
61 oxidation damage is the base excision repair (BER) pathway. In BER, DNA  
62 glycosylases are responsible for cleavage of the *N*-glycosidic bond between the base and  
63 the pentose sugar, removing modified DNA bases and creating an apurinic or  
64 apyrimidinic site (AP site). Endonucleolytic activity of the glycosylases or an AP-  
65 endonuclease transforms AP sites to gaps in DNA that are filled by a DNA polymerase  
66 and sealed by a DNA ligase [3,4]. In the present study we have evaluated effects of two  
67 phytochemicals found in fruits, vegetables and spices on DNA oxidation and DNA  
68 repair.

69 Accumulating evidence from epidemiological studies as well as laboratory data suggest  
70 that consumption of fruits and vegetables is associated with a reduced risk of  
71 developing a wide range of cancers including colon cancer [5,6]. Dietary strategies for  
72 cancer prevention are considered attractive alternatives because the consumption of  
73 natural compounds with potential chemopreventive effects is associated with low  
74 toxicity, safety and good acceptance by the public [7,8].

75 Ursolic acid (UA), a natural pentacyclic triterpenoid acid, is widespread in nature and  
76 abundant in certain medicinal plants. UA has been reported to possess a wide range of  
77 biological activities, such as anti-inflammatory, anticarcinogenic, antihyperglycemic,  
78 hepatoprotective and neuroprotective activities [9-12].

79 Luteolin (Lut) is a flavon, a subclass of flavonoids, found in fairly large amounts in  
80 fruits, vegetables, olive oil, red wine and tea. Many studies have shown that Lut exhibits  
81 a variety of pharmacological activities, including anti-inflammatory, antibacterial,

82 antioxidant and anticancer activities [13-16]. Contrarily to Lut, UA is not an antioxidant  
83 at relevant cellular redox conditions.

84 Protection of DNA from damage and modulation of DNA repair enzyme capacities may  
85 be assumed to contribute to protection against mutations and to maintenance of genomic  
86 stability. In the current study we evaluated DNA-protective and repair-enhancing effects  
87 of Lut and UA in human colon cells (Caco-2) exposed to oxidative agents. DNA  
88 damage was evaluated by alkaline single-cell gel electrophoresis (comet assay). BER of  
89 oxidised DNA was measured using an *in vitro* assay for incision activity of a cell  
90 extract, incubated with a substrate containing oxidised DNA bases [17]. We also  
91 assessed the ability of cells to rejoin strand breaks induced in DNA by H<sub>2</sub>O<sub>2</sub>. UA and  
92 Lut were used in concentrations likely to be attained in gut when humans have a diet  
93 rich in fruits and vegetables.

94

95

## 96 **2. Material and methods**

### 97 *2.1. Chemicals*

98 UA (purity  $\geq$  90%), hydrogen peroxide, Dulbecco's Modified Eagle Medium (DMEM),  
99 penicillin/streptomycin, trypsin solution and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl  
100 tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA).  
101 Lut (purity  $>$  90%) was from Extrasynthese (Genay, France). Fetal bovine serum (FBS)  
102 was purchased from Biochrom KG (Berlin, Germany). Ro (photosensitizer Ro19-8022)  
103 was from F.Hoffmann-La Roche (Basel, Switzerland). SYBR Gold (nucleic acid gel  
104 stain) was from Invitrogen Molecular probes (Oregon, USA). All other reagents and  
105 chemicals used were of analytical grade.

106

### 107 *2.2. Cell culture*

108 Caco-2 cells (derived from human colon carcinoma) were maintained as monolayer  
109 cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%  
110 FBS and antibiotics (100U/ml penicillin and 100 $\mu$ g/ml streptomycin), under an  
111 atmosphere of 5% CO<sub>2</sub> at 37°C. Cells were trypsinised when nearly confluent.

112 Cells were seeded onto 12-well plates, with 1 ml/well at a density of  $0.2 \times 10^6$  cells/ml,  
113 and incubated with different concentrations of test compounds in complete DMEM  
114 medium to test for possible direct cytotoxicity, genotoxicity, and for effects on induced  
115 DNA oxidation, and for modulation of DNA repair. Stock solutions of UA and Lut were  
116 prepared in dimethyl sulphoxide (DMSO) and aliquots kept at  $-20^{\circ}\text{C}$ . The final  
117 concentration of DMSO in medium was  $<0.5\%$ .

118

### 119 2.3. Cell toxicity assay

120 The test compound's cytotoxicity was assayed in 12-multiwell culture plates seeded  
121 with  $0.2 \times 10^6$  cell /well. Twenty-four hours after plating, the medium was discarded  
122 and fresh medium containing test compounds at different concentrations was added.  
123 After 48h of incubation with test compounds, cytotoxicity was evaluated by MTT test.  
124 The number of viable cells in each well was estimated by the cell capacity for reduction  
125 of MTT as described by [18]. The results were expressed as a percentage of cell  
126 viability relative to control (cells without any test compound).

127

### 128 2.4. Comet assay

129 The alkaline version of the single cell gel electrophoresis assay was used to evaluate  
130 DNA damage as previously described [19] with some modifications. Briefly, Caco-2  
131 cells were trypsinized, washed, centrifuged, and the pellet suspended in low melting  
132 point agarose; about  $2 \times 10^4$  cells were placed on a slide (pre-coated with 1% normal  
133 melting point agarose and dried), and covered with a coverslip. After 10 min at  $4^{\circ}\text{C}$ , the  
134 coverslips were removed and slides were placed in lysis solution (2.5M NaCl, 100mM  
135  $\text{Na}_2\text{EDTA}$ , 10mM Tris Base, pH 10 plus 1% Triton X-100) for 1h at  $4^{\circ}\text{C}$ . When  
136 oxidised bases were to be measured, after lysis slides were washed three times with  
137 buffer (40mM HEPES, 0.1M KCl, 0.5mM EDTA, 0.2mg/ml BSA, pH 8.0) and  
138 incubated with 30 $\mu\text{l}$  of formamidopyrimidine DNA glycosylase (FPG) in this buffer or  
139 with buffer only for 20 min at  $37^{\circ}\text{C}$ . Slides were then placed in horizontal  
140 electrophoresis chamber with electrophoresis solution (300mM NaOH, 1mM  
141  $\text{Na}_2\text{EDTA}$ , pH  $>13$ ) for 30 min at  $4^{\circ}\text{C}$  for the DNA to unwind before electrophoresis  
142 was run for 30 min at 25V and  $\sim 300\text{mA}$ . After electrophoresis, slides were washed two  
143 times with PBS and dried at room temperature. For analysis of the comet images, slides

144 were stained with SYBR Gold solution for 30 min at 4°C; after drying, slides were  
145 analysed in a fluorescence microscope and Comet 4 analysis system (Perceptive  
146 software) was used to calculate the parameter % tail intensity. Generally, 100 randomly  
147 selected cells are analyzed per sample.

148

#### 149 *2.5. Genotoxic effects of UA and Lut*

150 Caco-2 cells were incubated for 24h at 37°C with UA and Lut at different  
151 concentrations. Cells were collected by trypsinization and DNA damage (strand breaks,  
152 SBs) was evaluated by the alkaline version of the comet assay. Digestion with FPG  
153 allowed detection of oxidized purines [20].

154

#### 155 *2.6. Effects of UA and Lut on DNA oxidation.*

156 To evaluate protection against oxidative damage, Caco-2 cells were preincubated with 5  
157 and 10 µM UA or 10 and 20 µM Lut for 24h (long period of incubation) or 2h (short  
158 period of incubation) at 37°C. Cells were washed with PBS and treated with H<sub>2</sub>O<sub>2</sub> (75  
159 µM in PBS) for 5min on ice to induce SBs, or with 1µM Ro (photosensitizer Ro19-  
160 8022, prepared in PBS from a stock solution at 1 mM in ethanol) plus visible light from  
161 a 500 W tungsten-halogen source (1.5min on ice) at 33cm to induce 8-oxoGua. DNA  
162 damage (SBs and 8-oxoGua) was evaluated by the comet assay without or with FPG,  
163 respectively.

164

#### 165 *2.7. Effects of UA and Lut on cellular repair.*

166 In the cellular repair assay two different treatment regimes were used: First, pre-  
167 treatment with UA or Lut followed by exposure to H<sub>2</sub>O<sub>2</sub> and recovery in fresh medium.  
168 Caco-2 cells were preincubated with UA or Lut for 24h at 37°C. Cells were washed with  
169 PBS and treated with H<sub>2</sub>O<sub>2</sub> (75 µM) for 5 min on ice to induce SBs. The H<sub>2</sub>O<sub>2</sub> was  
170 removed and cells were washed with PBS and then incubated in fresh culture medium  
171 for 0, 10, 30 or 60 min at 37°C. Thus we evaluated the effect of pre-incubation in UA or  
172 Lut on the ability of cells to rejoin SBs [19]. In the second approach, to look for a  
173 possible direct effect of UA or Lut on enzyme activity, H<sub>2</sub>O<sub>2</sub> treatment was performed  
174 before cells were incubated with the test compounds. Briefly, Caco-2 cells were treated

175 with H<sub>2</sub>O<sub>2</sub> (75 μM) for 5 min on ice to induce SBs. Cells were washed with PBS to  
176 remove H<sub>2</sub>O<sub>2</sub> and then incubated with UA or Lut for 0, 10, 30 or 60 min at 37°C.  
177 Results were expressed as % of repair DNA damage that was calculated using the  
178 follow formula:

179 % of repair DNA damage =  $(T_0 - T_{30}) / (T_0 - C_{30}) \times 100$ ; where T<sub>0</sub> represents DNA  
180 damage before recovery period, T<sub>30</sub> represents DNA damage after 30 min of recovery  
181 and C<sub>30</sub> represents DNA damage of the control after 30 min of recovery.

182

### 183 2.8. *Effects of UA and Lut on BER (in vitro assay)*

184 This assay measures the excision repair activity of an extract prepared from cells treated  
185 with test compounds by providing the extract with a DNA substrate (agarose-embedded  
186 nucleoids) containing specific damage [17]. In this case, the substrate DNA was from  
187 cells previously exposed to Ro plus visible light to induce 8-oxoGua that is repaired by  
188 BER, and was prepared as described by Gaivão et al. [21]. Incision at damage sites,  
189 detected using the alkaline comet assay, indicates the capacity of glycosylase in the  
190 extract to initiate BER.

191

#### 192 2.8.1. *Cell extract preparation*

193 Extracts were prepared as described previously [17] with some modifications. Briefly,  
194 for extract preparation, Caco-2 cells were incubated with 10μM UA, 10μM Lut or 0.5%  
195 DMSO (control) for 24h at 37°C. Cells were washed with PBS, trypsinized and  
196 resuspended in PBS. Cells were divided into aliquots of 1x10<sup>6</sup> cells in 1ml and after  
197 centrifugation (14000g; 5min at 4°C) the dry pellets were frozen in liquid nitrogen and  
198 stored at -80 °C.

199

#### 200 2.8.2. *Substrate preparation*

201 Substrates for BER assay were prepared as described previously [17]. Briefly, HeLa  
202 cells cultivated in flasks, when near to confluence were treated with Ro plus visible  
203 light (5 min at 33cm on ice) to induce 8-oxoGua. Cells were washed with PBS,  
204 trypsinised and resuspended in medium. Cells were centrifuged, the pellet resuspended  
205 in freezing medium (DMEM medium supplemented with 20% FBS and 10% DMSO)

206 and aliquots of  $1 \times 10^6$  cells in 1ml frozen slowly and stored at  $-80^\circ\text{C}$ . HeLa cells without  
207 Ro treatment were also frozen in freezing medium and stored at  $-80^\circ\text{C}$ .

208

### 209 2.8.3. *Substrate incubation with cell extract*

210 On the day of the experiment, extracts were resuspended in 65  $\mu\text{l}$  of extraction buffer  
211 (45mM Hepes, 0.4M KCl, 1mM EDTA, 0.1mM dithiothreitol and 10% glycerol, pH  
212 7.8) plus Triton X-100 (0.25%), mixed 5sec on vortex at top speed and incubated 5 min  
213 on ice. After centrifugation ( $\sim 14,000 \times g$ ,  $4^\circ\text{C}$ , 5min) 55  $\mu\text{l}$  of supernatant was removed  
214 and mixed with 220 $\mu\text{l}$  of cold reaction buffer (40mM HEPES, 0.1M KCl, 0.5 mM  
215 EDTA and 0.2mg/ml bovine serum albumin, pH 8). Two gels per slide containing  $2 \times 10^4$   
216 substrate cells /gel (with or without treatment with Ro) were placed on slides pre-coated  
217 with normal melting point agarose and lysed for 1h. Slides were washed three times  
218 with reaction buffer and 30 $\mu\text{l}$  of extract was added to each gel and incubated 20 min at  
219  $37^\circ\text{C}$  in a moist box. FPG and reaction buffer were included as positive and negative  
220 controls, respectively. After incubation, slides were transferred immediately to alkaline  
221 electrophoresis solution and the normal comet assay was run [17, 22.]

222

### 223 2.9. *Statistical analysis*

224 Results were expressed as mean  $\pm$  SEM at least 3 independent experiments.  
225 Significant differences ( $P < 0.05$ ) were evaluated by Student's t-test.

226

## 227 **3. Results**

### 228 3.1. *Cytotoxic effects of UA and Lut*

229 In order to choose the concentrations of UA and Lut that can be used in protective  
230 studies, evaluations of test compounds' toxicity were done using MTT test. When Caco-  
231 2 cells were incubated for 48h, UA and Lut significantly decreased cell viability only at  
232 concentrations higher than 50 and  $100 \mu\text{M}$ , respectively (Fig. 1). For the follow  
233 experiments non-cytotoxic concentrations of UA and Lut were used.

234

### 235 3.2. *Genotoxicity of UA and Lut*



236 The effects of UA and Lut on induction of SBs and oxidized bases were evaluated. For  
237 this, Caco-2 cells were incubated with UA (5 and 10 $\mu$ M) or Lut (10 and 20 $\mu$ M) for 24 h  
238 at 37°C and DNA damage assessed by the comet assay with and without FPG treatment.  
239 At tested concentrations UA and Lut did not induce either SBs or oxidized purines  
240 (FPG-sensitive sites) (Fig.2).

241

### 242 3.3. *Effects of UA and Lut on oxidatively induced-DNA damage.*

243 To evaluate possible effects of UA or Lut on oxidatively induced-DNA damage, Caco-2  
244 cells were incubated for 24h (a long) or 2h (a short) periods with the compounds before  
245 treatment with H<sub>2</sub>O<sub>2</sub> or Ro.

246 Both 5 $\mu$ M UA and 20 $\mu$ M Lut, with a long period of incubation, significantly decreased  
247 DNA SBs induction by H<sub>2</sub>O<sub>2</sub> (Fig.3A). With a short period of incubation (2h), the  
248 effects of UA and Lut were even more pronounced (Fig.3B). The protective effects  
249 were not dose-dependent.

250 In the assay with Ro plus light, Caco-2 cells were also pre-treated for a long or short  
251 period with UA or Lut. With a long period of incubation, compounds at tested  
252 concentrations did not protect DNA from damage induced by Ro (Fig.4A). With a short  
253 incubation Lut significantly decreased oxidized DNA bases induced by Ro, while UA  
254 (10  $\mu$ M) showed a tendency to protect Caco-2 cells (Fig.4B).

255

### 256 3.4. *Effects of UA and Lut on repair ability.*

#### 257 3.4.1. *Cellular repair assay*

258 The ability of Caco-2 cells to rejoin strand breaks induced by H<sub>2</sub>O<sub>2</sub> was assessed by  
259 measuring damage remaining at different times of recovery (0, 10, 30 and 60 min). SBs  
260 decreased with the time of recovery and at 60 min the levels of SBs were similar to the  
261 control (without H<sub>2</sub>O<sub>2</sub> treatment) (data not shown). To assess effects of the test  
262 compounds on the ability of Caco-2 cells to rejoin DNA strand breaks, two different  
263 treatments were used. First, cells were treated with compounds for 24 h before H<sub>2</sub>O<sub>2</sub>  
264 exposure and recovery in fresh medium for 30 min at 37°C. For recovery time we chose  
265 30 min because it is within the linear phase of SB repair (Fig. 5A). Caco-2 cells treated  
266 only with H<sub>2</sub>O<sub>2</sub> (control cells), after 30 min of recovery had rejoined ~ 50% of SBs.

267 Cells pre-incubated with 5 $\mu$ M UA or 10 $\mu$ M Lut had rejoined 86% and 88%  
268 respectively, representing a relative increase in the extent of DNA rejoining of 65% and  
269 68% compared with the control cells, respectively. The highest concentrations of UA  
270 and Lut show a tendency ( $p \leq 0.1$ ) to increase the ability to rejoin SBs (Fig.5B).

271 In the second treatment, cells were incubated with test compounds for different times  
272 after H<sub>2</sub>O<sub>2</sub> exposure. No difference was found when cells were incubated with  
273 compounds during the recovery period when compared with cells incubated with fresh  
274 medium after H<sub>2</sub>O<sub>2</sub> exposure (data not shown) indicating the absence of any direct  
275 influence on repair enzymes.

276

### 277 3.4.2. BER activity measured *in vitro*

278 The ability of Caco-2 cells to repair oxidised bases by BER was measured by a  
279 modified comet assay, the *in vitro* BER assay. In this assay a DNA substrate containing  
280 specific damage, 8-oxoGua, induced by Ro plus visible light was incubated with an  
281 extract of Caco-2 cells (treated with UA or Lut for 24h at 37 °C). Figure 6 shows, first,  
282 that substrate when treated with FPG (positive control) increased SBs detected by comet  
283 assay compared with substrate incubated only with reaction buffer (negative control).  
284 Second, extract from Caco-2 cells treated only with DMSO led to an increase in SBs in  
285 substrate DNA when compared with the negative control. This means that the extract  
286 from Caco-2 cells has BER activity. And third, extracts obtained from cells pre-treated  
287 with 10  $\mu$ M UA showed significantly increased excision repair activity, by 24% when  
288 compared with an extract of Caco-2 cells treated with DMSO, while repair activity was  
289 not significantly affected by pre-treatment with Lut. There was no increase in SBs when  
290 extracts were incubated with substrate without 8-oxoGua (data not shown), indicating  
291 that the increase of breaks observed for UA corresponds to 8-oxoguanine DNA  
292 glycosylase 1 (OGG1) enzyme activity and confirming the absence of nonspecific  
293 nucleases in cell extracts.

294

## 295 4. Discussion

296 The integrity of DNA is critically important for DNA replication and cell division.  
297 Oxidative DNA damage in addition to a defective DNA repair mechanisms are known  
298 to be associated with carcinogenesis [23, 24]. Dietary antioxidants have the possibility

299 to prevent oxidation, but this requires that they are in proximity to the DNA in an active  
300 form. Several authors have reported that a compound's lipophilicity is a determinant  
301 characteristic for biological activity of the compounds. UA and Lut represent two  
302 classes of phytochemicals with different chemical and biological properties. Lut has free  
303 radical scavenging activity, whereas UA is virtually inactive as a free radical scavenger.  
304 Both are, however, highly lipophilic [25-27]. We evaluated the effects of both  
305 compounds, UA and Lut, against oxidative damage in Caco-2 cells at two levels: DNA  
306 protection and DNA repair. In this work, we show that ursolic acid and luteolin not  
307 only protect DNA from oxidative damage after a short period of pre-incubation but also  
308 increase repair activity in Caco-2 cells.

309 Concerning DNA protection, after a short incubation period (2 h) UA and Lut had a  
310 strong protective effect against H<sub>2</sub>O<sub>2</sub>-induced DNA damage. After a long period of  
311 incubation (24 h) both compounds showed a protective effect, but the percentage  
312 protection was smaller than with a short incubation period. In a previous paper, we  
313 showed that UA had chemoprotective activity against *t*BHP-induced DNA damage in  
314 HepG2 cells [24]. Our results are in agreement with other reports that also show that  
315 UA protects against H<sub>2</sub>O<sub>2</sub>-induced DNA damage [28,29] and decreased the level of  
316 AZT (3'-azido-3'-dideoxythymidine)-induced SBs in Caco-2 and HepG2 cells [30]. The  
317 protective effect of Lut against H<sub>2</sub>O<sub>2</sub>-mediated DNA SBs in Caco-2 cells is also in  
318 agreement with results obtained with other cell lines [26,31-35].

319 Besides DNA SBs, 8-oxoGua is one of the most abundant forms of oxidative damage  
320 and has been shown to cause G to T transversions. To evaluate effects on DNA  
321 protection against 8-oxoGua formation, we exposed Caco-2 cells to Ro plus visible light  
322 (to induce 8-oxoGua). Lut protected against Ro-induced DNA damage in Caco-2 after a  
323 short period of pre-incubation while UA showed a similar tendency. However, this  
324 protective effect was not observed with a long period of pre-incubation for either  
325 compound. Moon et al. [36] reported that dietary antioxidants such as quercetin, rutin  
326 and resveratrol as well as UA inhibit single strand breaks and 8-oxoGua in U937 cells  
327 exposed to 3-morpholinosydnomine N-ethylcarbamide (SIN-1). The protective effects  
328 of Lut against 8-oxoGua found in Caco-2 are in agreement with others authors. Cai et  
329 al. [37] showed that Lut, quercetin and genistein decrease oxidative damage to DNA,  
330 and among the test compounds, Lut had the most potent quenching effect on Fenton  
331 reaction-induced 8-oxoGua formation. Also Min and Ebeler [38] showed that several

332 flavonoids including Lut slightly inhibited 8-oxoGua formation in calf thymus DNA at  
333 low, physiologically relevant concentrations.

334 Phytochemicals such as flavonoids and triterpenoids can act as antioxidants in cells by  
335 modulating the activity of enzymatic and non-enzymatic cellular antioxidants and  
336 activating (phase I) enzymes and detoxifying (phaseII) enzymes involved in xenobiotic  
337 metabolism [24,39,40].

338 The protective effect of UA has been attributed to the ability of UA to increase levels of  
339 non-enzymatic antioxidants such as glutathione (GSH) and to increase the activity of  
340 antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPX) and  
341 superoxide dismutase (SOD) [29,41,42]. Martin-Aragon et al. [43] reported that UA  
342 restores hepatocyte antioxidant levels preventing carbon tetrachloride-induced liver  
343 damage. Also, Saravan et al. [44] showed that UA has a hepatoprotective effect against  
344 chronic ethanol-mediated toxicity in rats. UA increased levels of circulatory  
345 antioxidants such as reduced glutathione, ascorbic acid and alpha-tocopherol improving  
346 the antioxidant status of alcoholic rats.

347 Németh et al. [45] reported that Lut and quercetin were incorporated in small intestinal  
348 epithelial cells and located in the nuclei, decreasing 8-oxoGua formation. Lut has been  
349 reported as able to modulate antioxidant status, increasing the activities of antioxidant  
350 enzymes GPX, glutathione-S-transferase (GST), glutathione reductase (GR), SOD and  
351 CAT or attenuating the decrease of antioxidant levels (e.g. GSH) induced by toxic  
352 agents [14,26,46].

353 In our study Lut and UA seem to exert effects through cellularly mediated mechanisms  
354 that can be lost with time. Despite the differences of antiradical capacity between the  
355 two compounds, both showed a strong protector effect against oxidation of DNA,  
356 reinforcing the notions that cellularly mediated effects and the degree of hydrophobicity  
357 and consequently uptake into the cell are important factors to be taken into account  
358 when assessing the effectiveness of antioxidant protection.

359 DNA damage combined with defects in repairing oxidative damage to DNA has been  
360 associated with a development of several diseases including cancer [47,48]. Cells have  
361 multiple DNA repair pathways for specific classes of lesions that mitigate the  
362 deleterious consequences of damage accumulation. Effects of natural compounds on  
363 DNA repair are still poorly understood; some reports show that polyphenols such as

364 curcumin and quercetin increase DNA repair activity [27,49]. To our knowledge, there  
365 are no studies reporting the effects of UA and Lut on DNA repair activity in colon cells.

366 In our present study, 24 h of pre-treatment with UA or Lut increased the rate of  
367 rejoining of strand breaks in Caco-2 cells after treatment with H<sub>2</sub>O<sub>2</sub>. However, when  
368 cells were incubated with test compounds after H<sub>2</sub>O<sub>2</sub>-induced damage, no such effects  
369 were observed. This suggests an effect of the compounds on induction of repair activity  
370 not due to direct interactions between UA or Lut and the repair enzymes.

371 The major mechanism that cells use to repair oxidative damage lesions is the BER  
372 pathway. Here, we have measured the incision activity of a cell extract from Caco-2  
373 cells treated for 24 h with test compounds on a DNA substrate containing specific  
374 damage (8-oxoGua), to evaluate induction of BER activity. For the first time we report  
375 that UA, but not Lut, has a BER-inductive effect, increasing incision activity in Caco-2  
376 cells. In accordance with our results, Silva et al. [34] did not find effects of Lut on BER  
377 activity in neuronal cells. However, Leung et al. [50] found that Lut increased the  
378 mRNA expression of DNA base excision repair enzymes, such as hOGG1 and apurinic  
379 endonuclease in human lung carcinoma cells.

380 In summary, we demonstrated for the first time that UA and Lut not only protect DNA  
381 from oxidative damage but also increase repair activity in Caco-2 cells. These protective  
382 effects of UA and Lut may contribute to their anti-carcinogenic effects. Modulation of  
383 DNA repair by these compounds and other phytochemicals needs to be further explored.  
384 *In vivo* studies in animals or humans, making use of functional biomarker assays such as  
385 the comet assay can provide a better understanding of the potentially important impact  
386 of phytochemicals on DNA repair pathways and cancer prevention.

387

### 388 **Conflict of interest**

389 There are no conflicts of interest to report.

390

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394

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## Figure legends

Figure 1 – Effects of UA and Lut on cellular viability (as % of control) of caco-2 cells as measured by MTT test. Results are expressed as mean  $\pm$  SEM, of at least three independent experiments.

Figure 2 – DNA damage (SBs and FPG-sensitive sites) in Caco-2 cells treated for 24h with UA and Lut. Results are expressed as mean  $\pm$  SEM, of at least three independent experiments.

Figure 3 – Effects of 24h (A) or 2h (B) of treatment with UA or Lut on DNA damage induced by 75 $\mu$ M H<sub>2</sub>O<sub>2</sub> (5 min, on ice) in Caco-2 cells. Results are expressed as mean  $\pm$  SEM, of at least three independent experiments.

Figure 4 – Effects of 24h (A) or 2h (B) of treatment with UA or Lut on DNA damage induced by 1 $\mu$ M Ro19-8022 plus light (1.5min, on ice) in Caco-2 cells. Results are expressed as mean  $\pm$  SEM, of at least three independent experiments.

Figure 5 – Kinetic of SBs rejoining (A); and extent of repair of H<sub>2</sub>O<sub>2</sub>-induced damage in Caco-2 cells after preincubation with UA or Lut (B). Results are expressed as mean  $\pm$  SEM, of at least three independent experiments.

Figure 6 – *In vitro* DNA repair: incision by extracts from Caco-2 cells pre-incubated with 10 $\mu$ M of UA and Lut. Extracts were incubated for 20min with gel-embedded nucleoid DNA containing 8-oxoGua lesions. Results are expressed as mean  $\pm$  SEM, of four independent experiments.

Figure 1

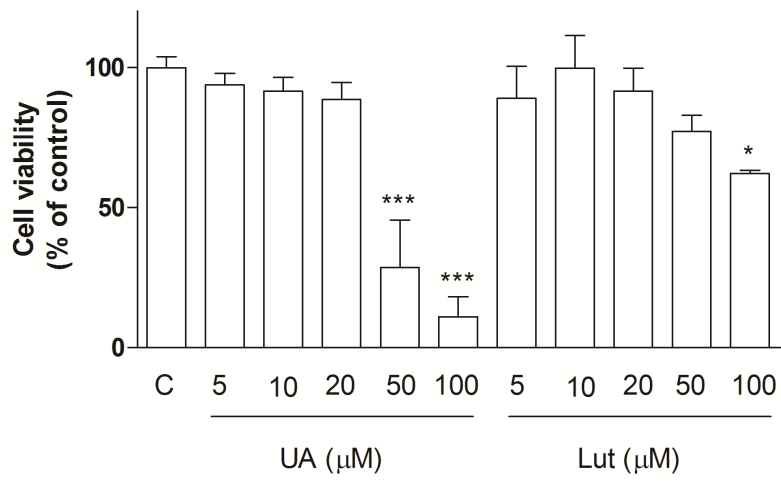


Figure 2

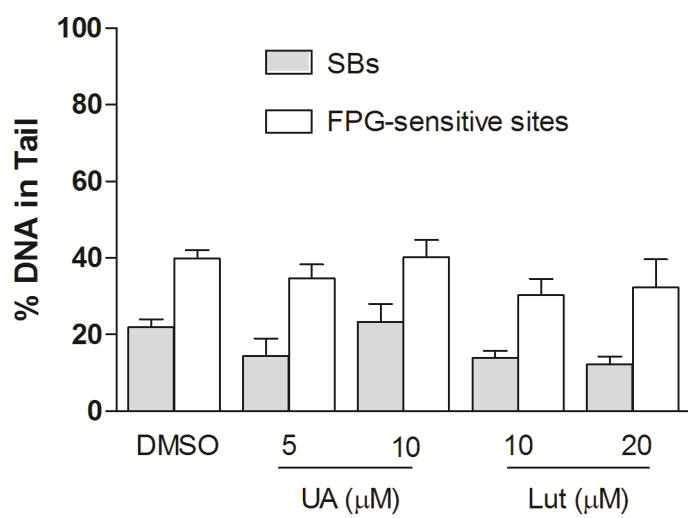


Figure 3

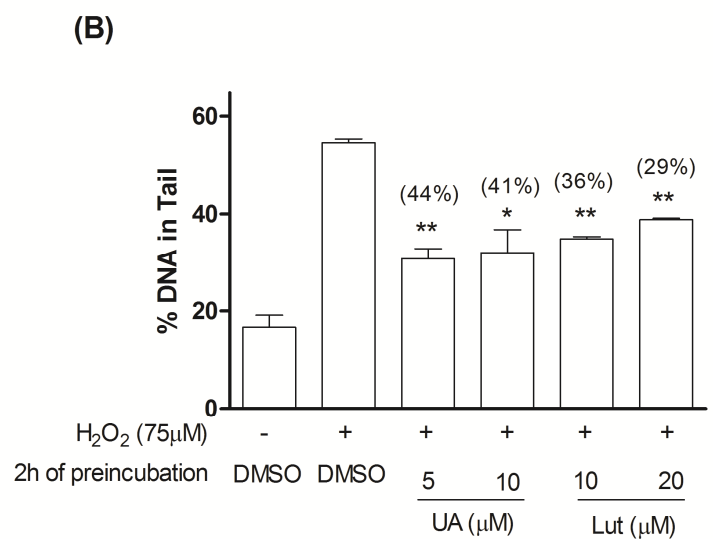
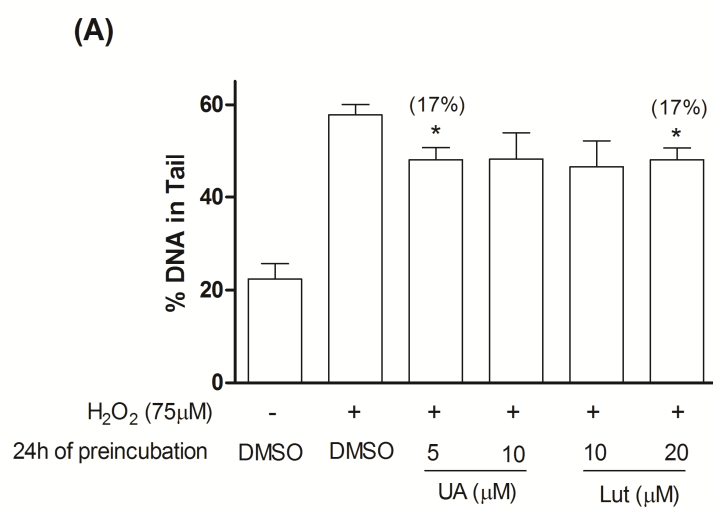
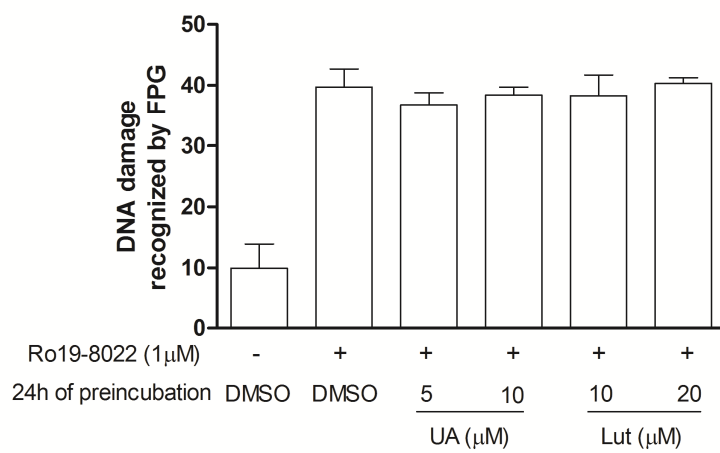


Figure 4

(A)



(B)

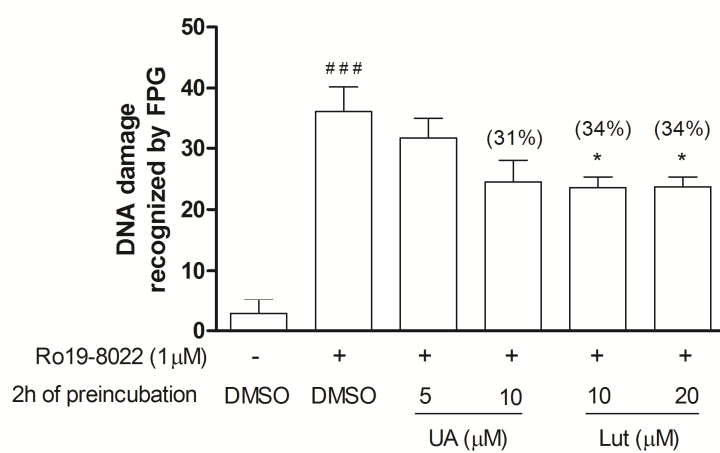


Figure 5

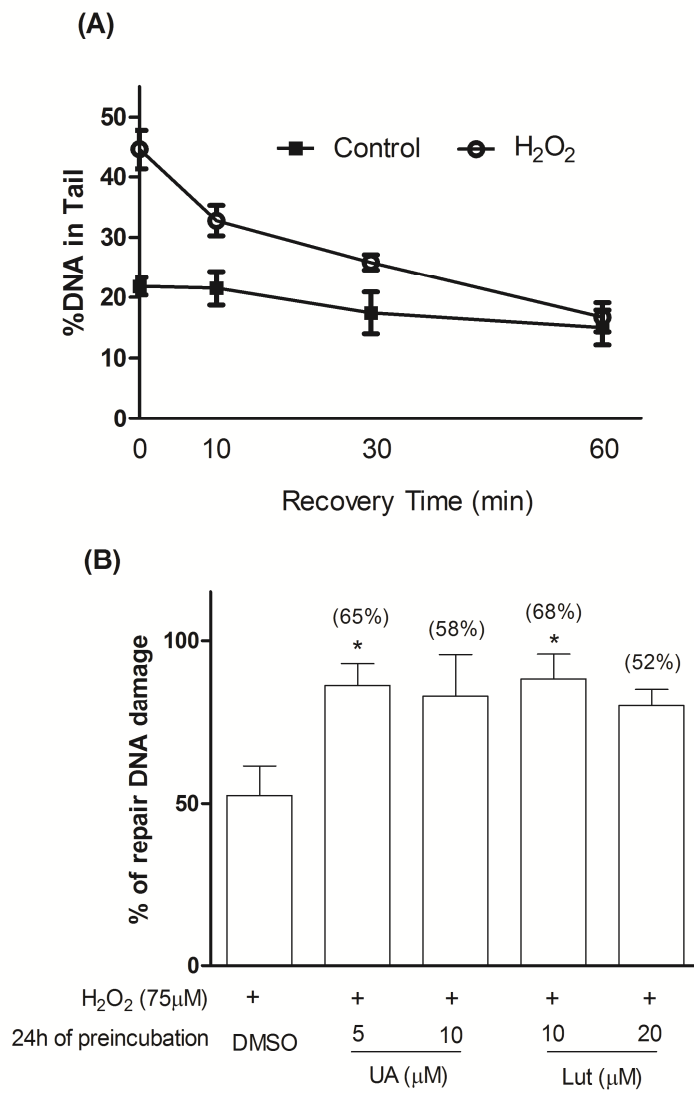


Figure 6

