

## Title

# **Luteolin, quercetin and ursolic acid are potent inhibitors of proliferation and inducers of apoptosis in both KRAS and BRAF mutated human colorectal cancer cells**

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1 **Abstract**

2 KRAS and BRAF mutations are frequent in colorectal carcinoma (CRC) and  
3 have the potential to activate proliferation and survival through MAPK/ERK and/or  
4 PI3K signalling pathways. Because diet is one of the most important determinants of  
5 CRC incidence and progression, we studied the effects of the dietary phytochemicals  
6 quercetin (Q), luteolin (L) and ursolic acid (UA) on cell proliferation and apoptosis in  
7 two human CRC derived cell lines, HCT15 and CO115, harboring **KRAS and BRAF**  
8 activating mutations, **respectively**. In KRAS mutated HCT15 cells, Q and L  
9 significantly decreased ERK phosphorylation, whereas in BRAF mutated CO115 cells  
10 the three compounds decreased Akt phosphorylation but had no effect on phospho-  
11 ERK. Our findings show that these natural compounds have antiproliferative and  
12 proapoptotic effects and simultaneously seem to act on KRAS and PI3K but not on  
13 BRAF. These results shed light on **the** molecular mechanisms of action of Q, L and UA  
14 and emphasize the potential of dietary choices for the control of CRC progression.

15

16 **Keywords:** Flavonoids, Ursolic acid, colorectal carcinoma, MAPK/ERK and PI3K  
17 pathways, molecular nutrition

## 18 Introduction

19 Colorectal carcinoma (CRC) is the third most common cancer worldwide. In  
20 particular sporadic CRC corresponding to 70-80% of total cases [1] is influenced by  
21 environmental factors, many of which diet related [2]. The mitogen-activated protein  
22 kinase/extracellular signal-regulated kinase (MAPK/ERK) and the phosphatidylinositol  
23 3-kinase (PI3K)/Akt are signalling pathways that have been implicated in oncogenic  
24 transformation in CRC. They confer a proliferative phenotype and resistance to therapy  
25 which is reflected in low patient survival [3-7]. Components of MAPK/ERK and  
26 PI3K/Akt pathways constitute, therefore, molecular targets for anticancer strategies [8-  
27 10]. Mutations of either KRAS (32%) or BRAF (14%) genes occur alternatively [11] in  
28 CRC, causing activation of either MAPK/ERK and PI3K pathways or MAPK/ERK  
29 pathway, respectively. Activation of MAPK/ERK pathway regulates the expression of a  
30 large number of proteins involved in the control of cell proliferation, differentiation and  
31 apoptosis [4, 12]. Activation of PI3K gene and inactivation of PTEN, common in CRC,  
32 result in overexpression of downstream targets, including Akt and PKC, which promote  
33 cell growth and rescue from apoptosis [13-17].

34 Epidemiological studies show that cancer incidence is inversely correlated with  
35 the consumption of diets rich in fruits and vegetables [18]. Natural compounds present  
36 in the diet, such as resveratrol and curcumin have been shown to be protective against  
37 cancer, contributing to decrease cancer risk and progression rate through their effects on  
38 signalling pathways related to proliferation and apoptosis [18, 19]. Studies in cell lines  
39 and animal models have shown that flavonoids inhibit cell proliferation and induce  
40 apoptosis in many types of cancer cells through different signalling pathways, which  
41 corroborate the suggestion that dietary choices may limit cancer progression [20-22].  
42 Quercetin (Q) and luteolin (L) (Figure 1) are two flavonoids found in fruits, vegetables

43 and aromatic plants with high antioxidant activity [23] to which anticancer properties in  
44 CRC are attributed [24-27]. In addition, a recent study showed that quercetin reduces  
45 the formation of aberrant crypt foci in a rat colon cancer induction model, suggesting  
46 the importance of this compound also in the prevention of colon cancer by decreasing  
47 cancer initiating events [28]. Although structurally related, the absence of the hydroxyl  
48 group at position 3 of L renders it more lipophilic than Q which may confer better  
49 access to intracellular targets. In agreement with this, we have previously shown that L  
50 is a more potent intracellular antioxidant than Q, and that this was related with its higher  
51 lipophilicity [23]. Ursolic acid (UA; Figure 1), a natural pentacyclic triperpenoid  
52 carboxylic acid, present ubiquitously in **plant foods** and also a major **constituent** in some  
53 medicinal plants possesses a wide range of biological activities, such as  
54 **hepatoprotective** and anti-inflammatory properties combined with low toxicity [29-31].  
55 However, contrarily to Q and L, UA is not an antioxidant at relevant cellular levels [32].  
56 Antitumor properties have also been attributed to UA and in colon cancer cells UA has  
57 been shown to induce apoptosis and inhibit proliferation [33-35]. Although potential  
58 effects on proliferation have been described for these compounds their effects on  
59 MAPK/ERK and PI3K pathways have not been established.

60 In spite of the general benefit of plant rich diets, variation in cancer incidence  
61 among individuals with similar dietary habits suggests interactions of food constituents  
62 with genetic factors [2, 18]. In the present study we report on the effects of Q, L and UA  
63 on two human derived cell lines which harbor different oncogene activating mutations,  
64 representative of a large number of CRC: HCT15 has a KRAS (G13D) mutation [36]  
65 whereas CO115 has a BRAF (V599E) mutation [37]. These mutations impact on  
66 MAPK/ERK and PI3K pathways. The relevance of effects on both these pathways for  
67 successful cancer treatment has recently been emphasized [38]. CRC remains a human

68 malignant tumor often resistant to available treatment and knowledge of anticancer  
69 properties of dietary constituents may guide dietary choices for cancer patients with  
70 particular genetic backgrounds and possibly also suggest their use in combination with  
71 conventional therapy in order to enhance therapeutic effects [39, 40].

72

### 73 **Material and methods**

74

#### 75 **Reagents and antibodies**

76 Quercetin (Q), ursolic acid (UA), wortmannin (W) and 3-(4,5-Dimethylthiazol-  
77 2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St.  
78 Louis, MO, USA). Luteolin (L) was from Extrasynthese (Genay, France) and PD-98059  
79 (PD) from Calbiochem (San Diego, CA). All other reagents and chemicals used were of  
80 analytical grade. Stock solutions of Q, L and UA were made in dimethyl sulfoxide  
81 (DMSO) and aliquots kept at -20°C.

82 Primary antibodies were purchased from the following sources: Cell Signaling  
83 (Danvers, MA, USA) the anti-p44/42 MAPK (ERK1/2) total, anti-phospho-Akt  
84 (Ser473), anti-Akt total, anti-phospho-PTEN (Ser380/Thr382/383) and anti-PTEN total;  
85 Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) the anti-phospho-ERK1/2, Raf-  
86 B and K-Ras; and Sigma-Aldrich the anti- $\beta$ -actin. Secondary antibodies HRP donkey  
87 anti-rabbit and sheep anti-mouse were purchased from GE Healthcare (Bucks, UK).

88

#### 89 **Cell culture**

90 HCT15 and CO115 human colon carcinoma-derived cell lines were maintained  
91 at 37°C in a humidified 5% CO<sub>2</sub> atmosphere in RPMI-1640 medium (Sigma-Aldrich)  
92 supplemented with 10mM HEPES, 0.1mM pyruvate, 1% antibiotic/antimycotic solution

93 (Sigma-Aldrich) and 10% fetal bovine serum (FBS; EU standard, Lonza, Verviers,  
94 Belgium). Cells were seeded onto six (2ml) and twelve (1ml) well plates at a density of  
95  $0.75 \times 10^5$  (HCT15) and  $1.0 \times 10^5$  (CO115) cells/ml. Incubations with different  
96 concentrations of test compounds were made in serum free medium for 48h for MTT  
97 test, BrdU incorporation and TUNEL assay, and for 24h (Akt, ERK and PTEN) or 6h  
98 (BRAF and KRAS) for western blot analysis.

99

#### 100 **Assessment of cell toxicity/proliferation by MTT reduction test**

101 A MTT reduction assay of the tested compounds was performed in order to  
102 select concentrations that were not cytotoxic and significantly inhibited cell  
103 proliferation. Cells were treated with test compounds for 46h before the 2h incubation  
104 with MTT (final concentration 0.5 mg/ml). Hydrogen chloride 0.04M in isopropanol  
105 was then added to dissolve the formazan crystals. The number of viable cells in each  
106 well was estimated by the cell capacity to reduce MTT. The results were expressed as  
107 percentage relative to the control (cells without any test compound). MTT reduction at  
108 the beginning of incubation (t=0h) was subtracted from all the experimental conditions  
109 used above, including the control. Since the effects of the compounds were studied  
110 after 48h of incubation and cells grow significantly within this time period, the point at  
111 the beginning of the incubation allows to distinguish between cell death and inhibition  
112 of proliferation. MTT negative values indicate necrotic cell death due to cytotoxicity.  
113 Values between 0% and 100% indicate inhibition of cell proliferation.

114

#### 115 **Assessment of proliferation by BrdU incorporation**

116 After 45h of treatment with test compounds at the chosen concentrations, cells  
117 were incubated with bromodeoxyuridine 10 $\mu$ M (BrdU; Sigma-Aldrich) for another 3h.

118 Both adherent and non-adherent cells were collected from each sample, fixed with 4%  
119 paraformaldehyde for 15min at room temperature and attached into a polylysine treated  
120 slide using a Shandon Cytospin (Thermo Fisher Scientific Inc, Waltham MA, USA).

121 Cells were incubated with HCl 2M for 20min, washed in PBS containing 0.5%  
122 Tween-20 and 0.05% BSA (TPBS-B) and then incubated with monoclonal mouse anti-  
123 BrdU antibody (DakoCytomation, Glostrup, Denmark) for 1h at room temperature.  
124 After washing in TPBS-B, cells were incubated with anti-mouse IgG FITC conjugated  
125 secondary antibody (Sigma-Aldrich) for 1h at room temperature, washed again and then  
126 incubated with Hoechst for nuclei staining. The percentage of proliferating cells was  
127 calculated as the ratio between BrdU positive cells and total cell number (nuclei staining  
128 with Hoechst) from a count higher than 500 cells per slide under a **fluorescence**  
129 microscope. Results are presented as mean  $\pm$  SEM of at least three independent  
130 experiments.

131

### 132 **Assessment of apoptosis by TUNEL assay**

133 Cells treated with the tested compounds at chosen concentrations for 48h were  
134 collected (both floating and attached cells) and fixed with 4% paraformaldehyde for  
135 15min at room temperature and attached into a polylysine treated slide using a Shandon  
136 Cytospin. Cells were washed in PBS and permeabilized with 0.1% Triton X-100 in  
137 0.1% sodium citrate for 2min on ice. TUNEL (TdT mediated dUTP Nick End  
138 Labelling) assay was performed using a kit from Roche (Mannheim, Germany),  
139 following the manufacture's instructions. Cells were incubated with Hoechst for nuclei  
140 staining. The percentage of apoptotic cells was calculated from the ratio between  
141 TUNEL positive cells and total number of cells (nuclei staining with Hoechst), from a

142 count higher than 500 cells per slide under a **fluorescence** microscope. Results are  
143 presented as mean  $\pm$  SEM of at least three independent experiments.

144

#### 145 **Protein extraction and western blotting**

146 After treatment with the chosen concentration of **test** compounds, cells were  
147 washed with PBS and lysed for 15min at 4°C with ice cold RIPA buffer (1% NP-40 in  
148 150mM NaCl, 50mM Tris (pH 7.5), 2mM EDTA), supplemented with 20mM NaF,  
149 1mM phenylmethylsulfonyl fluoride (PMSF), 20mM Na<sub>2</sub>V<sub>3</sub>O<sub>4</sub> and protease inhibitor  
150 cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using a  
151 Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and BSA  
152 used as a protein standard. Twenty micrograms of total protein from each cell lysate  
153 were separated by SDS gel electrophoresis and then electroblotted to a Hybond-P  
154 polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in  
155 TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) non-fat dry milk or BSA  
156 (bovine serum albumin), washed in TPBS and then incubated with primary antibody.  
157 After washing, membranes were incubated with secondary antibody conjugated with  
158 IgG horseradish peroxidase and immunoreactive bands were detected using the  
159 Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence  
160 detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity  
161 was quantified using the Quantity One software from Bio-Rad.  $\beta$ -actin was used as  
162 loading control. Results are presented as mean  $\pm$  SEM of at least three independent  
163 experiments.

164

#### 165 **Statistical analysis**



166 One-way ANOVA followed by the Student-Newman-Keuls test was used to  
167 perform statistical analysis, using GraphPad Prism 4.0 software (San Diego, CA, USA),  
168 and  $P$ -values  $\leq 0.05$  were considered statistically significant.

169

## 170 **Results**

171

### 172 **Effects of Q, L and UA on cell proliferation**

173 In order to choose doses that do not cause significant toxicity (**necrosis**) and  
174 have antiproliferative effects **on** the two human CRC derived cell lines, HCT15 and  
175 CO115, a MTT assay was performed with different concentrations of the tested  
176 **compounds. The results showed that necrotic cell death (negative values in Figure 2)**  
177 **occurred in HCT15 cells with L at 20 $\mu$ M and in CO115 with UA at 15 $\mu$ M.** All the  
178 tested compounds inhibited cell proliferation in a concentration-dependent manner in  
179 both cell lines as shown by MTT assay (Figure 2). Both reference inhibitors,  
180 wortmannin (W) and PD-98059 (PD), at the higher tested concentration did not induce  
181 cell toxicity. Based on MTT results, three concentrations of Q, L and UA (2 in case of  
182 CO115 cells) that inhibited cell proliferation without significant toxic effects, were  
183 selected and used in the following BrdU and TUNEL assays.

184 For the BrdU incorporation assay, cells were treated with compounds for **48h**.  
185 **As** shown in Figure 3, a significant inhibition of proliferation indicated by lower levels  
186 of BrdU incorporation was observed for Q, L and UA in both cell lines, in a dose  
187 dependent manner. In HCT15 cells, the levels of BrdU incorporation decreased from  
188 22.0% in the control to 5.2%, 3.4% and 9.8% in cells treated with Q 20 $\mu$ M, L 15 $\mu$ M  
189 and UA 4 $\mu$ M, respectively (Figure 3a). In CO115 cells, the percentage of cell  
190 proliferation significantly decreased from 19.1% in the control to 3.8%, 11.1% and

191 10.2% in cells treated with Q 15 $\mu$ M, L 12 $\mu$ M and UA 10 $\mu$ M, respectively (Figure 3b).  
192 The structurally-related flavonoids, Q and L, **showed different responses in the** two cell  
193 lines: L was a stronger proliferation inhibitor in HCT15 than in CO115, whereas Q was  
194 more effective in CO115 than in HCT15. The reference inhibitors, W, a PI3K inhibitor,  
195 and PD, a phospho-ERK inhibitor, significantly inhibited cell proliferation in HCT15  
196 and CO115 cells, respectively (Figure 3).

197

### 198 **Effects of Q, L and UA on apoptosis**

199 The ability of the test compounds to induce apoptosis was addressed by the  
200 TUNEL assay. As shown in Figure 4, all compounds significantly induced apoptosis in  
201 both HCT15 and CO115 cells. Apoptotic cells in HCT15 increased from 0.3% in the  
202 control to 4.4%, 3.9% and 6.6% in cells treated with the higher concentrations of Q, L  
203 and UA, respectively (Figure 4a). In CO115 cells, apoptotic cells increased from 1.9%  
204 in the control to 36.2%, 15.1% and 12.4% in cells treated with the higher concentrations  
205 of Q, L and UA, respectively (Figure 4b). Between the two flavonoids, Q seems to be a  
206 more potent inducer of apoptosis in both cell lines compared to L. In addition, UA  
207 showed to be the most effective in HCT15, where it induced 20 times more apoptosis at  
208 4 $\mu$ M when compared with control.

209

### 210 **Effects of Q, L and UA on ERK phosphorylation**

211 Activation of MAPK/ERK pathway is representative of a large number of CRC  
212 cases and the phosphorylation of ERK is an indicator of this activation. We observed  
213 high levels of phospho-ERK in both cell lines (Figure 5). Incubations with L (15 $\mu$ M)  
214 and Q (20 $\mu$ M) significantly decreased phospho-ERK protein level in HCT15 cells  
215 (Figure 5a), but not in CO115 cells, while **UA did** not have any effect **on** either of the

216 cell lines (Figure 5a,b). A significant reduction of phospho-ERK by PD, a reference  
217 inhibitor of MAPK/ERK pathway, was observed in both cell lines. Interestingly, L was  
218 a stronger inhibitor of ERK in HCT15 cells than the reference inhibitor PD and than the  
219 structure-related compound Q.

220

### 221 **Effects of Q, L and UA on Akt phosphorylation**

222 Because MAPK/ERK and PI3K/Akt pathways are both activated by RAS, we  
223 also checked if the PI3K/Akt pathway was affected by the **test** compounds, measuring  
224 phospho-Akt and phospho-PTEN expression levels. In HCT15 cells, there were no  
225 detectable amounts of phospho-Akt, in incubations with and without serum (Figure 6a).  
226 High expression levels of phospho-PTEN were detected in HCT15 cells but **were** not  
227 altered by the **test** compounds (data not shown). In CO115 cells, phospho-Akt  
228 expression was observed and significantly decreased by Q (15 $\mu$ M), L (12 $\mu$ M) and UA  
229 (10 $\mu$ M), as shown in Figure 6b. Wortmannin, a reference PI3K inhibitor, also  
230 significantly decreased Akt phosphorylation, contrarily to PD that did not alter  
231 phospho-Akt levels in CO115 cells. Phospho-PTEN and total PTEN expression were  
232 not observed in CO115 cells, in medium with and without serum, in contrast with what  
233 was observed with HCT15 cells (Figure 6c). **The lack of phospho-Akt in HCT15 and**  
234 **PTEN signal in CO115 cells was reproducible and checked in the presence of a positive**  
235 **reactive sample** (Figure 6a,c). These observations seem, therefore, also not to be the  
236 result of protein degradation during protein extraction or sample preparation.

237

### 238 **Effects of Q, L and UA on KRAS and BRAF expression**

239 To check if the effects of the tested compounds reflect direct effects on KRAS or  
240 BRAF, expression of these proteins was also monitored by western blot. As shown in

241 figure 7, Q and L remarkably decreased the expression of KRAS but not BRAF in both  
242 cell lines. UA significantly changed the expression of KRAS only in HCT15 cells, but  
243 not as efficiently as the flavonoids. No significant changes were induced by UA in  
244 BRAF expression.

245

## 246 Discussion

247 The effects of quercetin (Q), luteolin (L) and ursolic acid (UA), natural  
248 compounds common in diets rich in fruits and vegetables, were studied in two different  
249 human colon carcinoma-derived cell lines representative of common CRC cases. We  
250 observed that the three tested compounds, at concentrations that did not induce  
251 significant cell toxicity (necrosis), inhibited proliferation and induced apoptosis in both  
252 cell lines in a concentration-dependent manner. The purpose of the present study was to  
253 identify in HCT15 and CO115 cells molecular targets for Q, L and UA related with  
254 their antiproliferative and proapoptotic effects. HCT15 and CO115 have activating  
255 mutations of KRAS and BRAF, respectively.

256 Q and L decreased the expression of phospho-ERK in the KRAS mutated  
257 HCT15 cell line but not in the BRAF mutated CO115 cell line. These results suggest  
258 that the BRAF mutation in CO115 cells overrides any inhibitory effect of Q and L on  
259 phospho-ERK, indicating that these flavonoids act on KRAS upstream of BRAF (Figure  
260 8). This was further confirmed by a decrease in the expression of KRAS but not BRAF  
261 induced by both flavonoids. Our findings corroborate recent reports where quercetin  
262 treatment resulted in a reduction of Ras protein levels in colon cell lines expressing  
263 oncogenic Ras [41, 42]. A recent study in skin epidermal cell line, showed a different  
264 effect of quercetin, which in these cells inhibited both Raf and MEK activity [43]. In  
265 addition, we observed in HCT15 cells that Q and L decreased phospho-ERK levels as

266 efficiently as PD-98059 (PD), a specific inhibitor of MEK downstream of RAF [44].  
267 Inhibition of proliferation and induction of apoptosis by Q and L in HCT15 cells does  
268 however not seem to be due to phospho-ERK inhibition alone since PD inhibited  
269 phospho-ERK but was without effect on cell proliferation and induction of apoptosis.  
270 This finding is in agreement with other reports [10, 45], which showed that tumor cells  
271 carrying KRAS mutation do not rely only on MAPK/ERK pathway to proliferate. Since  
272 HCT15 cell proliferation was inhibited by wortmannin (W), a PI3K inhibitor, it seems  
273 that inhibition of proliferation by Q and L treatment could be through inhibition of PI3K  
274 dependent pathways. Contrarily to a previous report [46], we did not detect phospho-  
275 Akt in HCT15 cells, which could be explained by the high levels of phospho-PTEN  
276 observed (Figure 8). It is known that other downstream targets of PI3K besides Akt also  
277 contribute to cell proliferation and apoptosis, such as PKC, which is known to be  
278 inhibited by Q and L [47-51]. PKC isozymes have been shown to be commonly  
279 deregulated in colon cancer and other natural compounds, such as curcumin, have also  
280 shown to inhibit PKC in CRC cells [17]. Thus, inhibition of proliferation in HCT15  
281 cells by Q and L seems to be due to effects on KRAS, affecting not only the  
282 MAPK/ERK pathway but also other alternative pathways, such as PI3K/PKC pathway.  
283 However, apoptosis induced by Q and L in HCT15 cells does not seem to be due to  
284 inhibition of PI3K, since W did not induce apoptosis in this cell line. Other apoptotic  
285 **targets** of these compounds should be considered.

286         Studies have shown that BRAF mutated cell lines rely more on MAPK/ERK  
287 pathway for proliferation than KRAS mutated cell lines [10, 45]. However, our results  
288 show that the dependence of CO115 cells on MAPK/ERK pathway for proliferation is  
289 not exclusive, since all tested compounds inhibit proliferation without affecting  
290 phospho-ERK levels. CO115 cells, besides **harbouring a** BRAF mutation, **also present**

291 high PI3K activity [52]. In agreement with this, a high expression of phospho-Akt was  
292 observed while PTEN was not detectable. Our results show a significant decrease in  
293 phospho-Akt expression by Q, L and UA. These suggest an inhibition of PI3K activity,  
294 in addition to an inhibition of KRAS (Figure 8). Several studies have shown that  
295 quercetin and analogs are potent inhibitors of PI3K activity [53, 54]. However, W  
296 although inhibiting phospho-Akt, did not inhibit proliferation or induce apoptosis. The  
297 effect of the natural compounds on PI3K may, therefore, only partially explain their  
298 antiproliferative and proapoptotic activities in CO115 cells. The inhibition of phospho-  
299 Akt and phospho-ERK by Q and L has been reported in human hepatoma cell line  
300 (HepG2) and brain tumors [55-57].

301       Regarding UA, our results show that this compound does not affect phospho-  
302 ERK expression, being only effective in inhibiting phospho-Akt in CO115 cells. It  
303 seems that UA does not affect significantly KRAS (although it decreased expression  
304 levels in HCT15 cells) and has PI3K as one of its molecular targets. Interestingly, it was  
305 the most efficient proliferation inhibitor and inducer of apoptosis in HCT15 cells, which  
306 do not express phospho-Akt. Contrarily to the effects of the antioxidants Q and L, the  
307 antitumor properties of UA through redox-sensitive pathways are most likely not the  
308 result of its reactive oxygen species scavenging ability, since it was previously shown  
309 that UA is inactive as free radical scavenger [32]. Interestingly, all tested compounds  
310 showed to be more efficient than the reference compounds, PD and W, in inhibiting cell  
311 proliferation and inducing apoptosis. Their wider range of molecular targets is therefore  
312 advantageous in the control of tumor progression [26] and their importance of  
313 modulating several signal transduction pathways associated with carcinogenesis is once  
314 again reinforced.

315 In summary, the antiproliferative and proapoptotic effects of Q and L seem to  
316 be, at least in part, due to effects on KRAS through regulation of both MAPK/ERK and  
317 PI3K pathways. The BRAF mutation overrides the compounds` inhibition of KRAS on  
318 the MAPK/ERK pathway but not on the PI3K pathway. UA seems to act on PI3K  
319 where Q and L may also act, independently of KRAS mutation. The results of this study  
320 suggest, therefore, the applicability of these phytochemicals in dietary strategies and as  
321 possible adjuvants in CRC therapy both in KRAS and BRAF gene mutation profiles.

322

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## Figure legends

**Fig. 1.** Chemical structures of quercetin (Q), luteolin (L) and ursolic acid (UA).

**Fig. 2.** Effect of different concentrations of quercetin (Q), luteolin (L) and ursolic acid (UA), for 48h on MTT reduction in HCT15 (a) and CO115 (b) cells. Wortmannin (W) and PD-98059 (PD) were used as reference inhibitors of PI3K/Akt and MAPK/ERK pathways, respectively. Values are mean  $\pm$  SEM of at least 3 independent experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$  and \*\*\* $P \leq 0.001$  when compared to control.

**Fig. 3.** Effect on cell proliferation of different concentrations of quercetin (Q), luteolin (L), ursolic acid (UA) and reference compounds, wortmannin (W) and PD-98059 (PD), for 48h in HCT15 (a) and CO115 (b) cells, using the BrdU incorporation assay. Values are mean  $\pm$  SEM of at least 3 independent experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$  and \*\*\* $P \leq 0.001$  when compared to control.

**Fig. 4.** Effect on apoptosis of different concentrations of quercetin (Q), luteolin (L) and ursolic acid (UA) and reference compounds, wortmannin (W) and PD-98059 (PD), for 48h in HCT15 (a) and CO115 (b) cells, using the TUNEL assay. Values are mean  $\pm$  SEM of at least 3 independent experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$  and \*\*\* $P \leq 0.001$  when compared to control.

**Fig. 5.** Effects of quercetin (Q), luteolin (L) and ursolic acid (UA) for 24h on phospho-ERK expression in HCT15 and CO115 cells, using western blot.  $\beta$ -Actin was used as loading control. (a) HCT15 cells were treated with L 15 $\mu$ M (L15), Q 20 $\mu$ M (Q20) and

UA 4  $\mu$ M (UA4) in serum free medium. (b) CO115 cells were treated with 12 $\mu$ M L (L12), 15 $\mu$ M Q (Q15) and 10 $\mu$ M UA (UA10) in serum free medium. Wortmannin 1 $\mu$ M (W1) and PD-98059 50 $\mu$ M (PD50) were used as reference inhibitors of PI3K and MEK, respectively, in both cell lines. Values are mean  $\pm$  SEM of at least 3 independent experiments. \* $P \leq 0.05$  and \*\* $P \leq 0.01$  when compared to control.

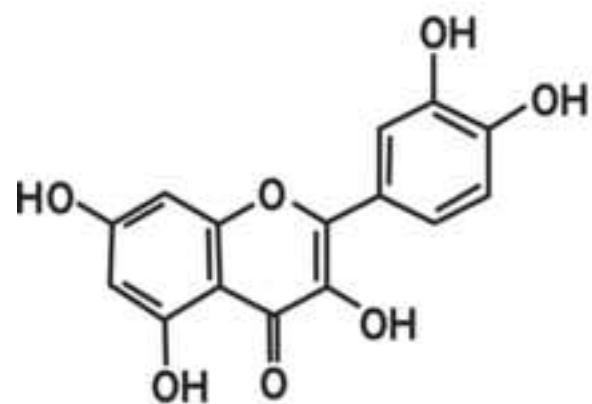
**Fig. 6.** Effects of quercetin (Q), luteolin (L) and ursolic acid (UA) for 24h on phospho-Akt expression and phospho-PTEN levels in HCT15 and CO115 cells, using western blot.  $\beta$ -Actin was used as loading control. (a) HCT15 cells were cultured in medium without and containing 2% or 10% serum (FBS) and CO115 cells were cultured in serum free medium. (b) CO115 cells were treated with UA 10 $\mu$ M (UA10), Q 15 $\mu$ M (Q15) and L 12 $\mu$ M (L12) in serum free medium. Wortmannin 1 $\mu$ M (W1) and PD-98059 50 $\mu$ M (PD50) were used as reference inhibitors of PI3K and MEK, respectively. (c) CO115 cells were cultured in medium without and containing 2% or 10% FBS and HCT15 cells were cultured in serum free medium. Values are mean  $\pm$  SEM of at least 3 independent experiments. \*\*\* $P \leq 0.001$  when compared to control.

**Fig. 7.** Effects of quercetin (Q), luteolin (L) and ursolic acid (UA) for 6h on KRAS and BRAF expression in HCT15 (a) and CO115 (b) cells, using western blot.  $\beta$ -Actin was used as loading control. Images and values are representative of three independent experiments.

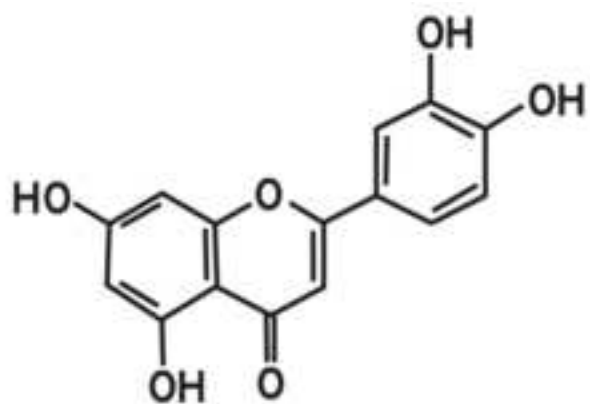
**Fig. 8.** Schematic representation of possible targets of quercetin (Q), luteolin (L) and ursolic acid (UA) on MAPK/ERK and PI3K/Akt pathways in HCT15 and CO115 cell lines, which may lead to inhibition of cell proliferation and induction of apoptosis.



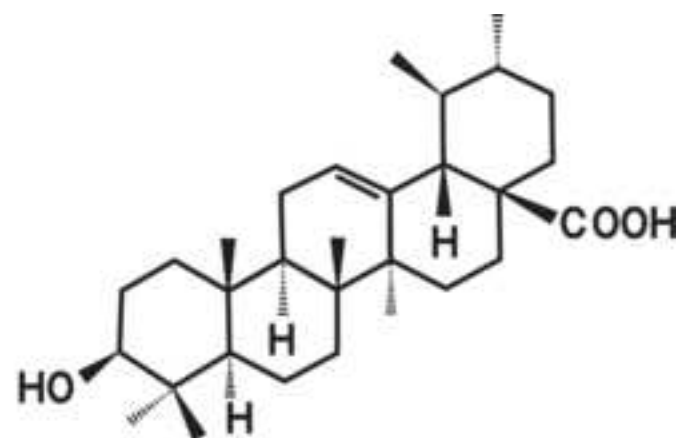
Figure 1  
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**Quercetin**



**Luteolin**

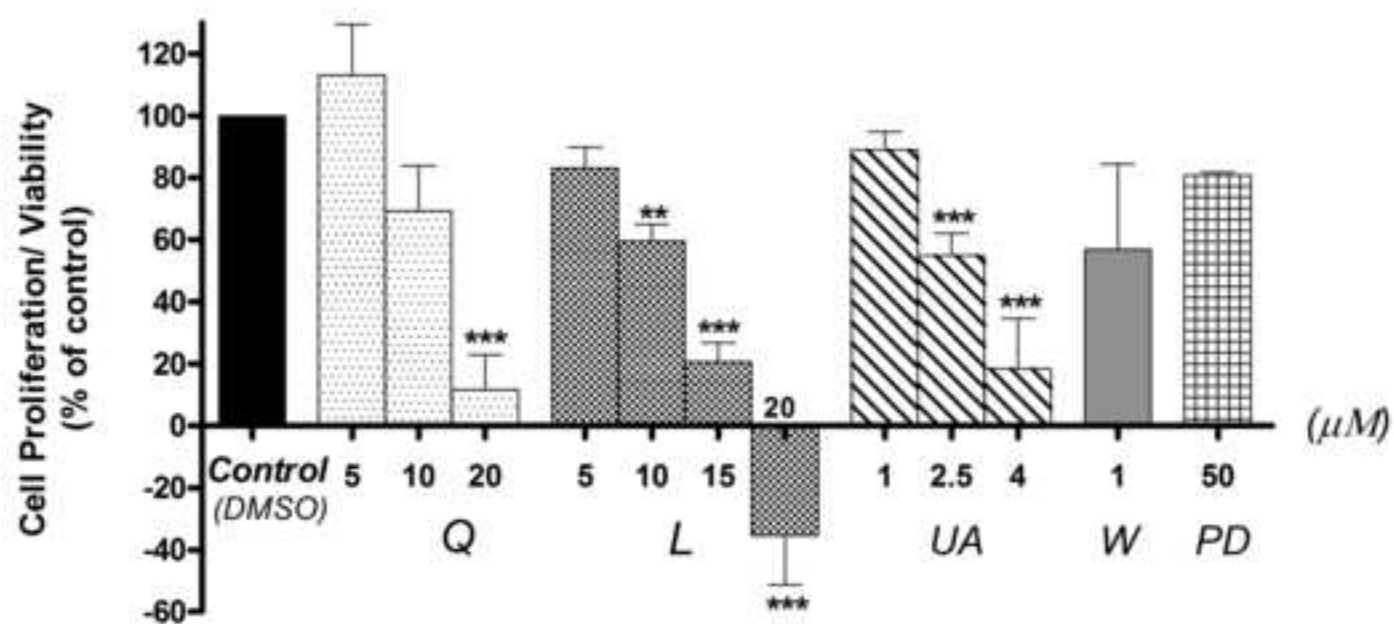


**Ursolic acid**

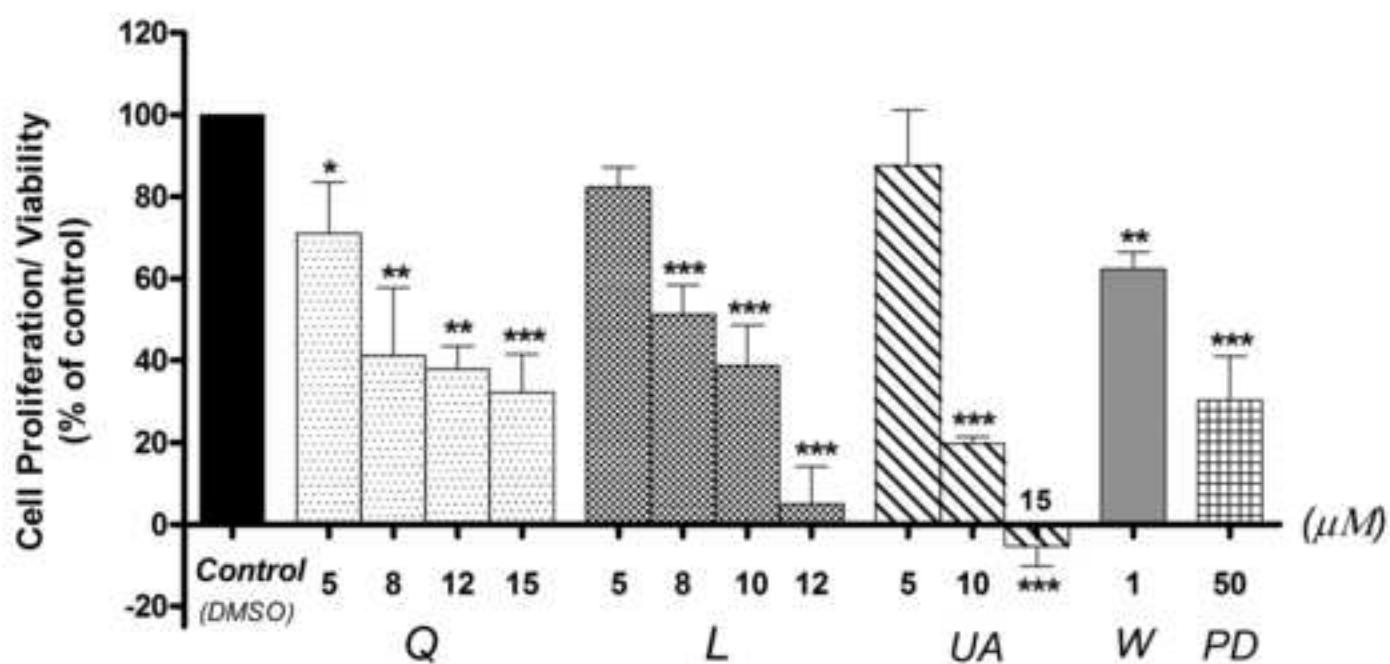
Figure 2

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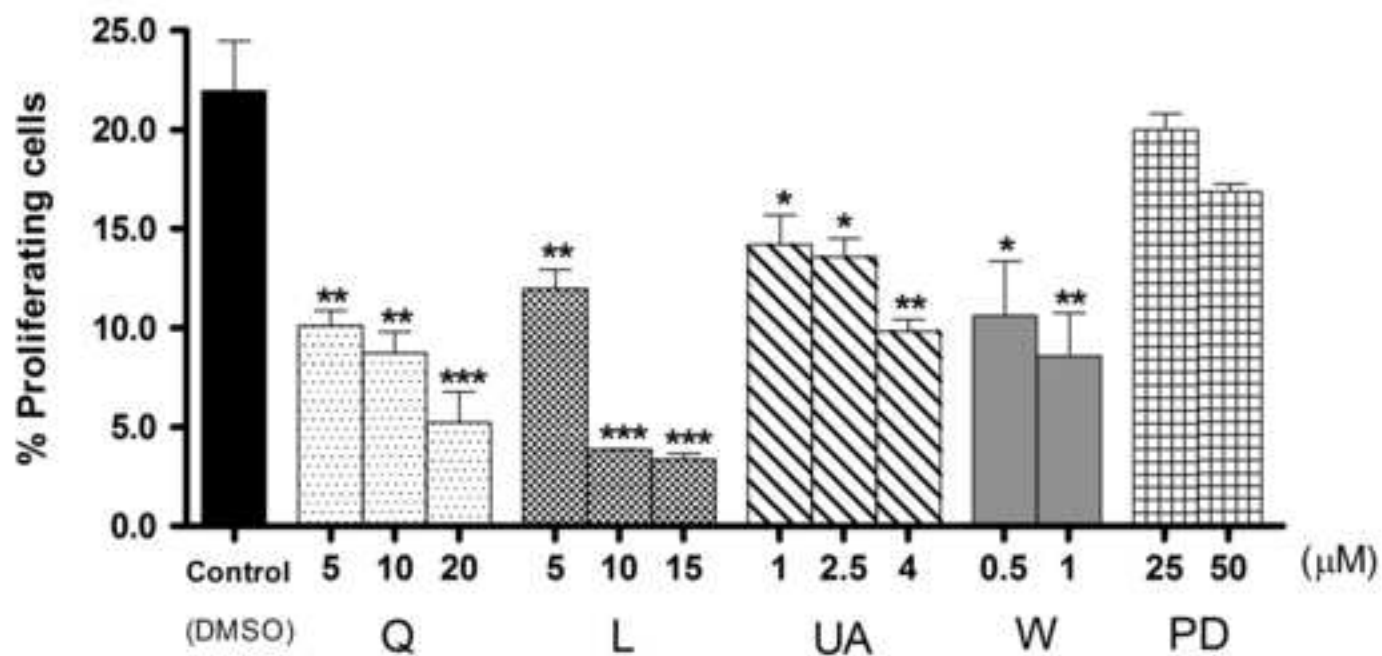
**a** MTT reduction test in HCT15



**b** MTT reduction test in CO115



**a** BrdU incorporation in HCT15



**b** BrdU incorporation in CO115

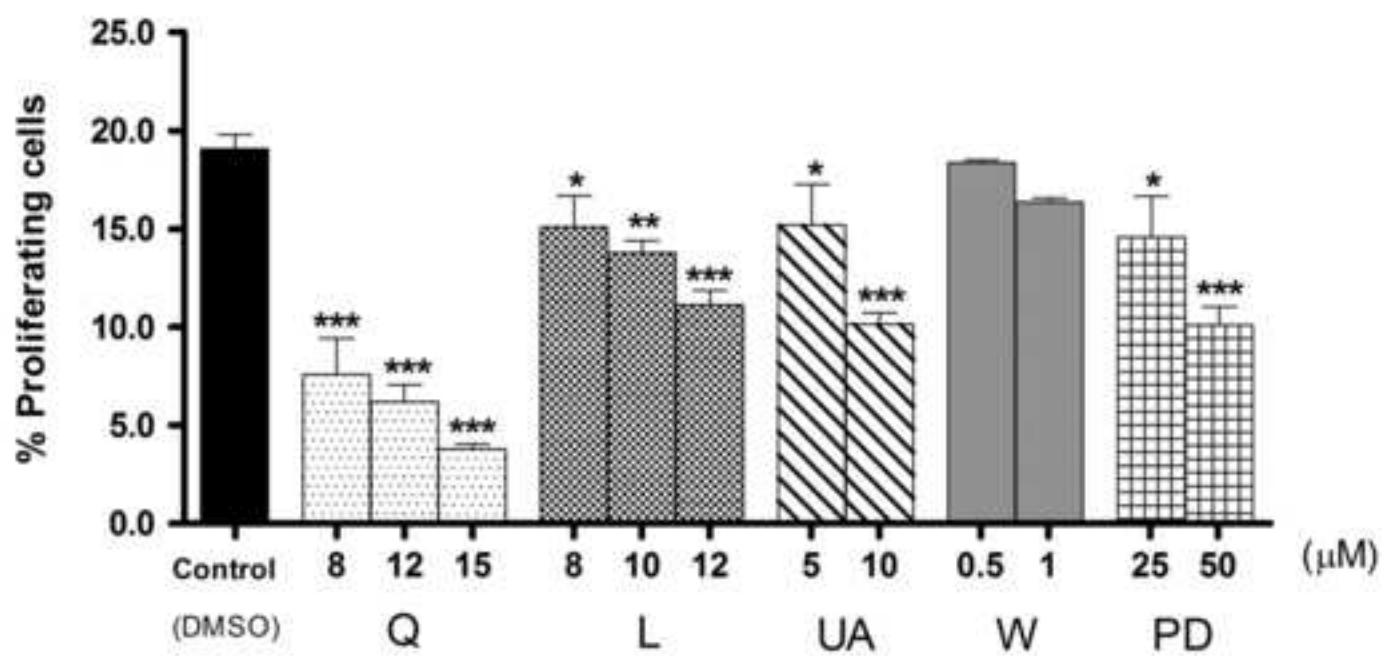


Figure 4  
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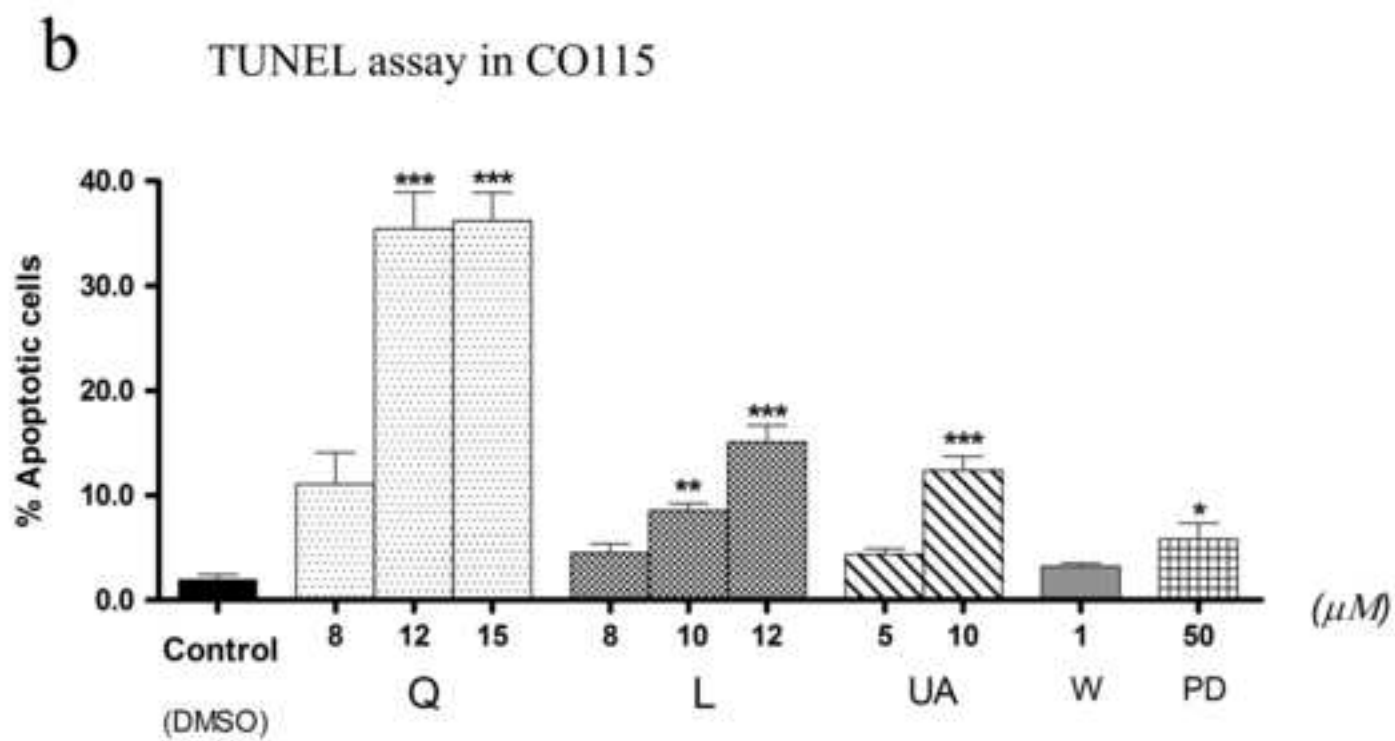
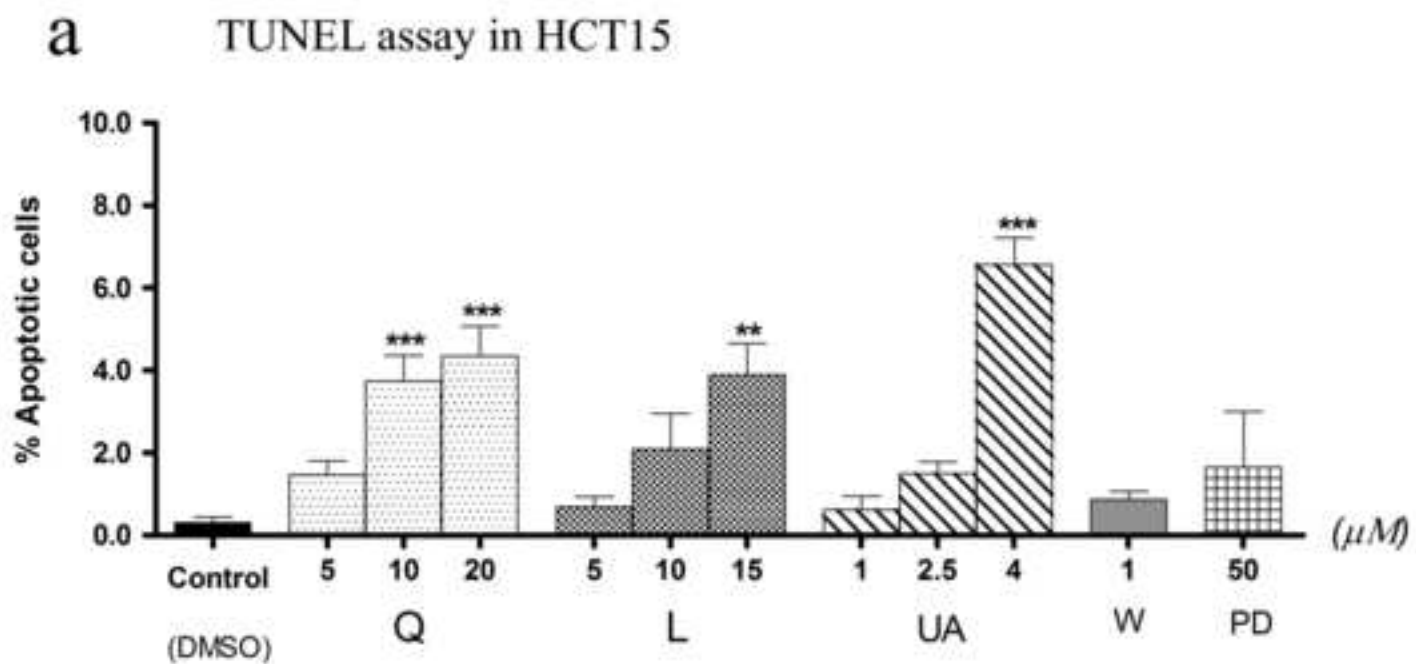


Figure 5  
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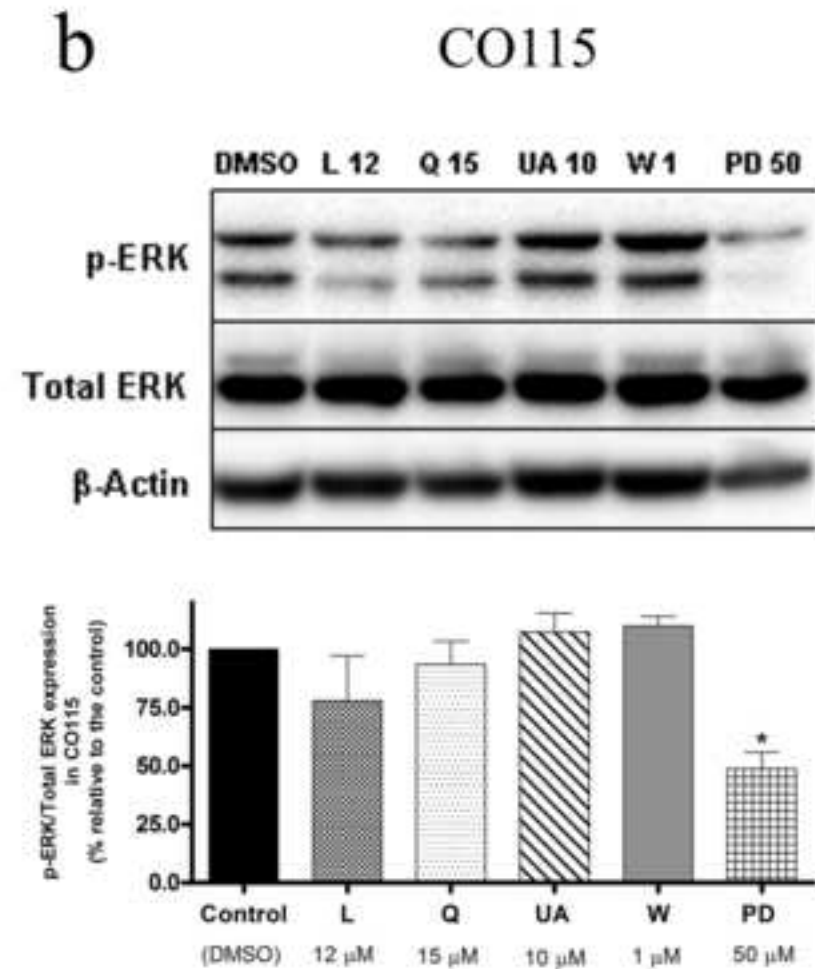
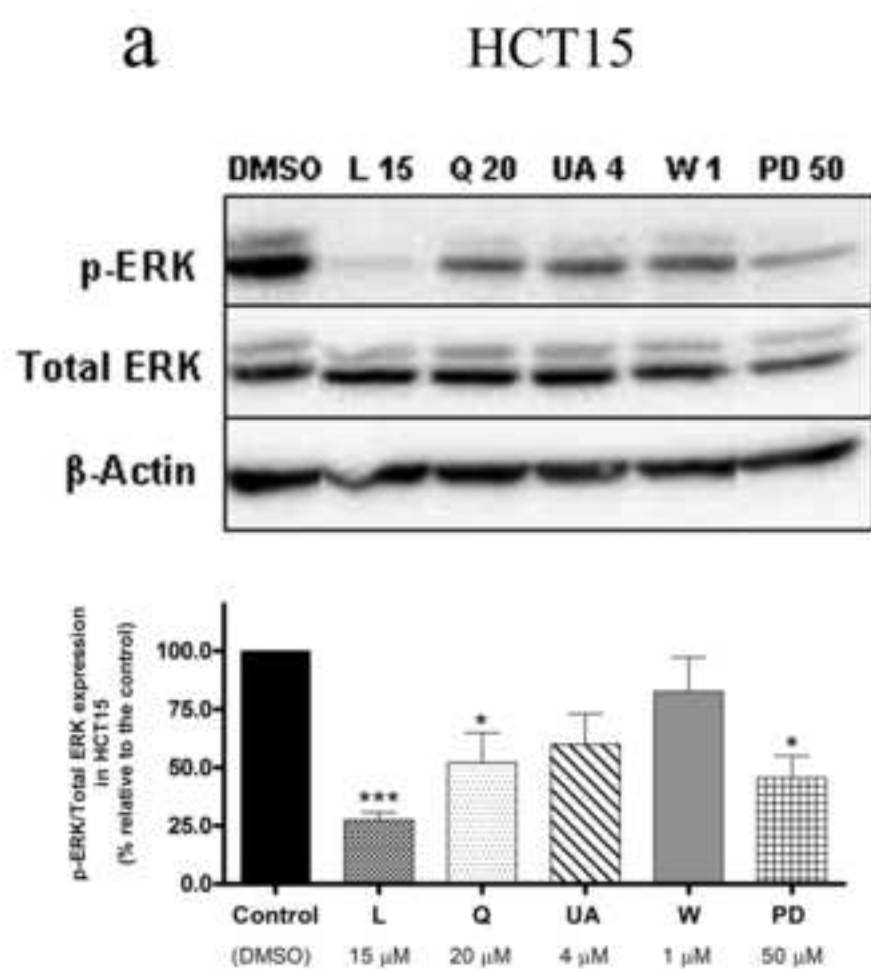


Figure 6  
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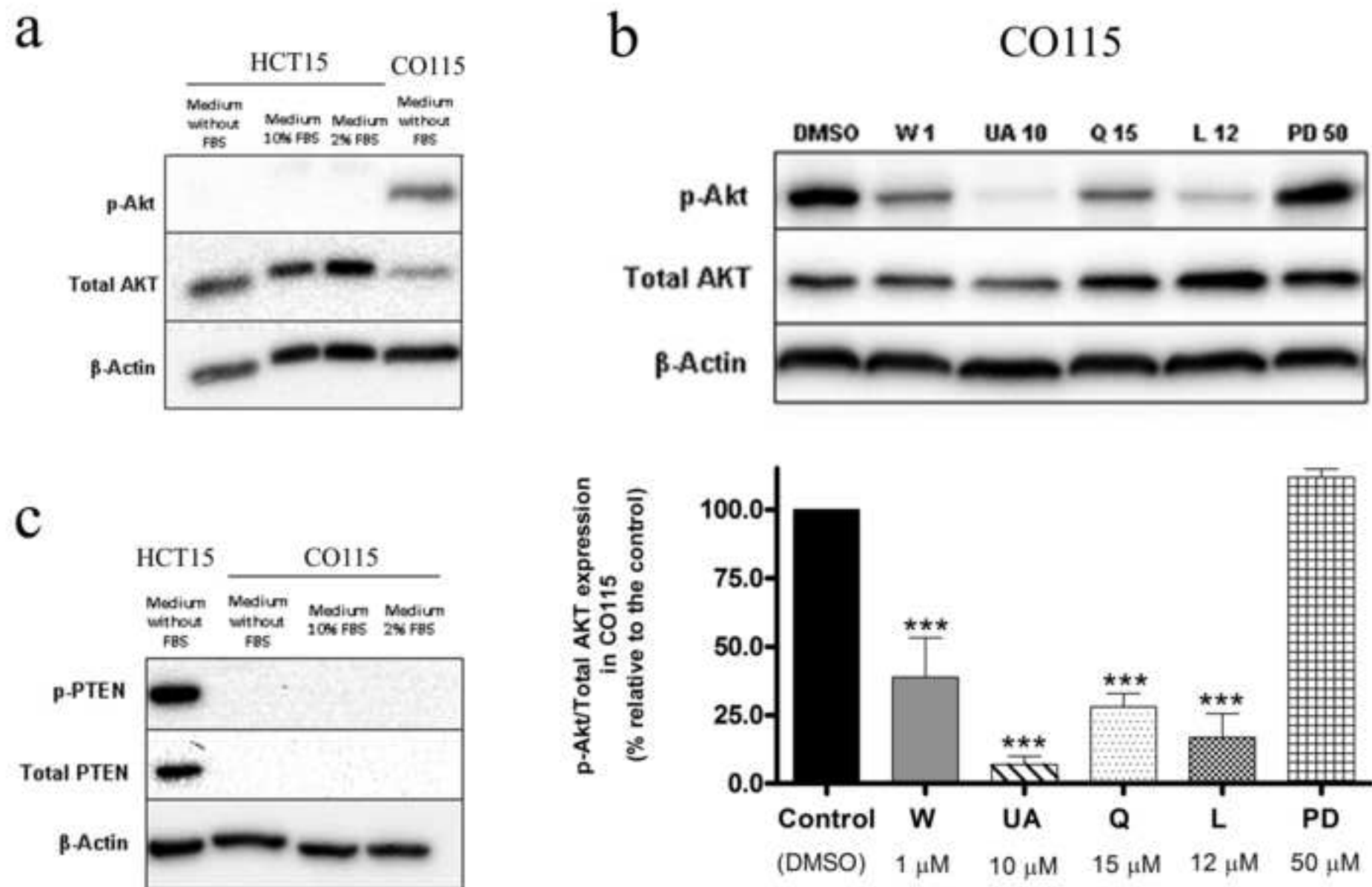




Figure 7  
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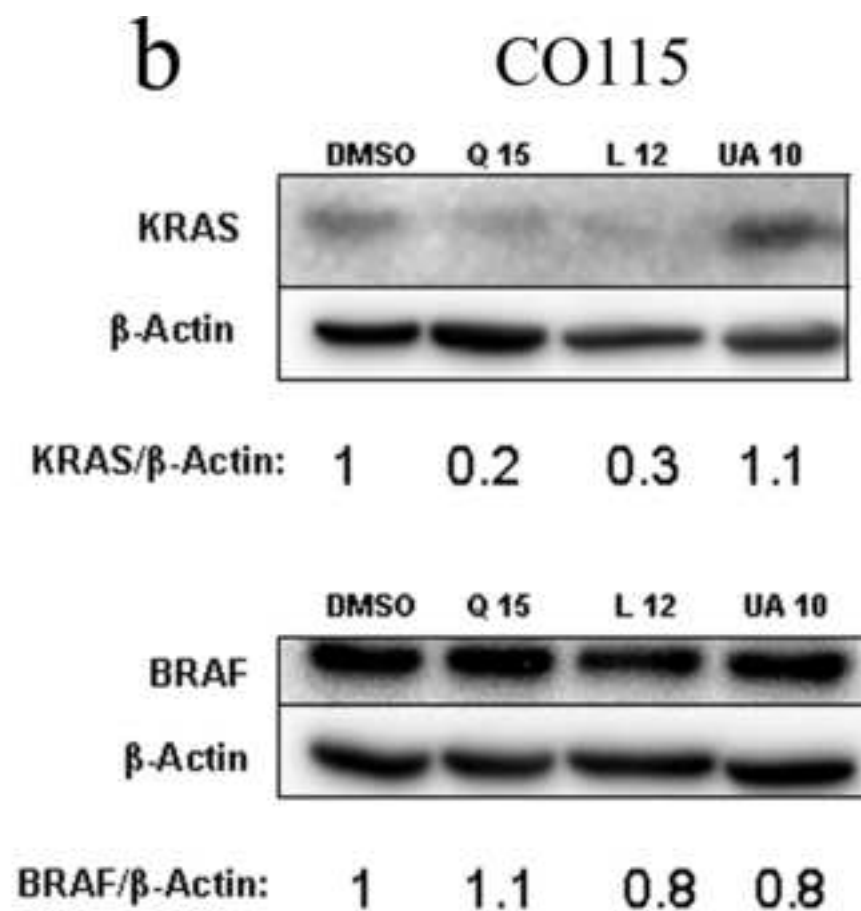
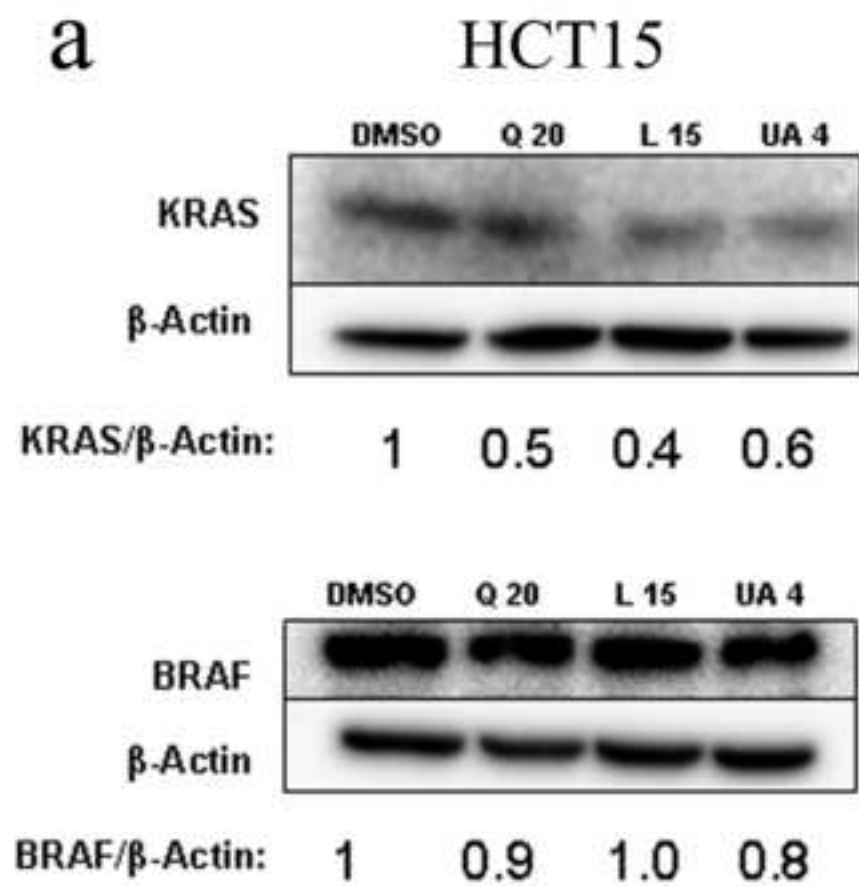
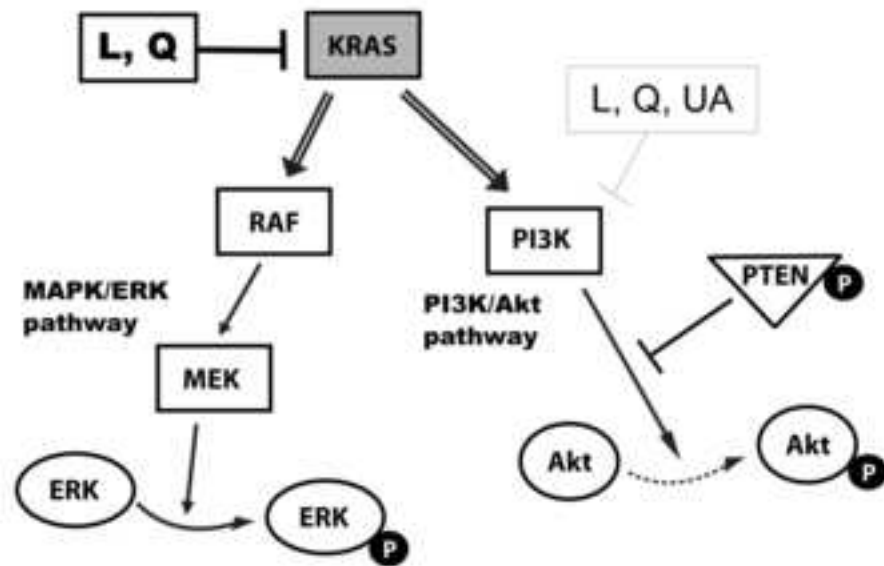


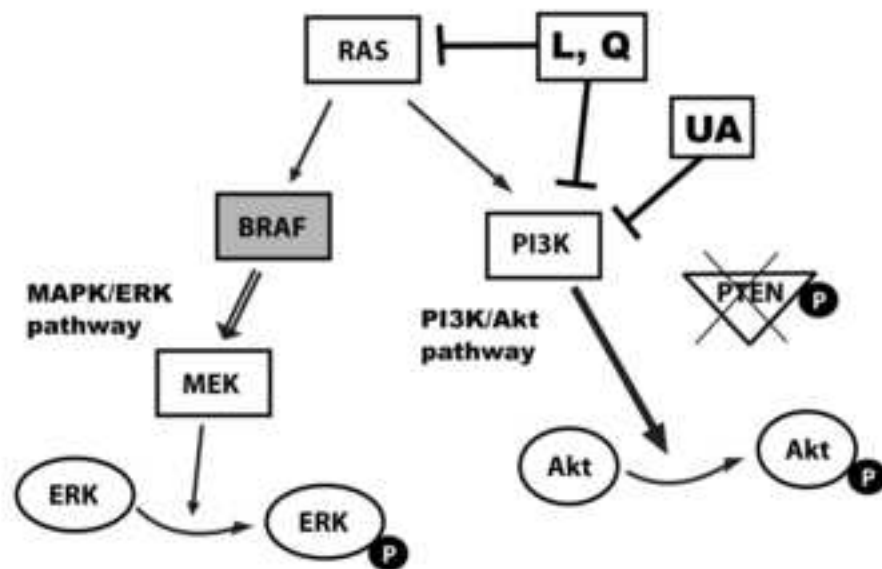
Figure 8

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## HCT15




## CO115



 Activating mutation       Abnormal activation

 Mutagenic constitutive activation

 Intrinsic low phosphorylation rates



## Conflicts of Interest Statement

The authors do not have conflicts of interest.