RSC Publishing Food & Function

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Journal:	*Food & Function*
Manuscript ID:	FO-ART-10-2011-010226.R1
Article Type:	Paper
Date Submitted by the Author:	n/a
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Hypericum androsaemum water extract inhibits proliferation in human colorectal cancer cells through effects on MAP kinases and PI3K/Akt pathway

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Abstract

MAP kinase and PI3K/Akt signalling pathways are commonly altered in colorectal carcinoma (CRC) leading to tumor growth due to increased cell proliferation and inhibition of apoptosis. Several species of the genus *Hypericum* are plants used in Portugal to prepare herbal teas to which digestive tract effects are attributed. In the present study, the antiproliferative and proapoptotic effects of the water extracts of *H. androsaemum* (HA) and *H. perforatum* (HP) were investigated in two human colon carcinoma-derived cell lines, HCT15 and CO115, which harbour activating mutations of KRAS and BRAF, respectively. Contrarily to HP, HA significantly inhibited cell proliferation and induced apoptosis in both cell lines. HA decreased BRAF and phospho-ERK expressions in CO115, but not in HCT15. HA also decreased Akt phosphorylation in CO115 and induced p38 and JNK in both cell lines. HA induced cell cycle arrest at S and G2/M phases as well as caspase-dependent apoptosis in both cell lines. Chlorogenic acid (CA), the main phenolic compound present in the HA extract and less represented in the HP water extract, did, however, not show any of those effects when used individually. In conclusion, water extract of HA, but not of HP, controlled CRC proliferation and specifically acted on mutant and not wild-type BRAF. This effect of HA was, however, not due to CA alone.

Keywords: BRAF, Chlorogenic acid, Colorectal Carcinoma, *Hypericum androsaemum*, *Hypericum perforatum*, MAP kinases, PI3K/Akt Pathway

Introduction

Environmental factors, many of which diet related, are responsible for 70-80% of total cases of colorectal carcinoma (CRC), an important health problem worldwide (1, 2). To the two species of *Hypericum* (family Hypericaceae), *Hypericum androsaemum* (HA) and *Hypericum perforatum* (HP), used in this study (spontaneous in the north of Portugal) important medicinal properties have been attributed. *H. perforatum* (HP), also known as St. John's wort, is the most studied of *Hypericum* species and it is known for its pharmacological antidepressant activities and its antiviral and antibacterial properties (3). The anticarcinogenic activity of HP has also been reported in several cancer cell types, although not in CRC cells (4-7). Its antitumor effects have been related with one of its main constituents, hypericin (8-10), that is, however, residual in the water extract (11). *H. androsaemum* is less studied but it is the most frequently used in Portugal due to its diuretic, hepatoprotector, cholagogue, and also anti-kidney failure properties, as well as in the relief of digestive tract disorders (12, 13). Its anticarcinogenic activity has, to our knowledge, never been reported. Recent *in vitro* studies showed the antioxidant and hepatoprotective activities of HA water extract (14, 15). The effects of HA have been attributed, at least in part, to the presence of several flavonoids, such as quercetin and its glycosides, and phenolic acids, such as chlorogenic acid (15). Chlorogenic acid (CA) is distinctively more abundant in HA water extract than in HP water extract. HA water extract does not posses hypericin in its constitution (11). Plants containing a variety of phenolic compounds have been shown to play an important role as dietary antioxidants in cancer prevention (16, 17). However, evidence is increasing that the anticarcinogenic properties of plant food constituents is not only the result of their antioxidant activity. In fact, many of these constituents have been demonstrated to act

on multiple key elements in signalling pathways related to cellular proliferation and apoptosis (18, 19).

Activating mutations of KRAS, BRAF and/or PI3K have been found in more than 50% of CRC cases and constitutively activate the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and/or the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathways (20, 21). The constitutive activation of these pathways results in higher cell proliferation rates and in inhibition of apoptosis (20, 22-24). Since MAPK/ERK and PI3K/Akt pathways are involved in CRC progression and drug resistance, proteins associated with these pathways are good molecular therapeutic targets for drug discovery (25, 26). Also, the stress-activated protein kinases, c-Jun N-terminal kinase (JNK) and p38, are two other major MAPK pathways frequently deregulated in cancer, including CRC (26, 27). They are involved in the control of cell proliferation and apoptosis, therefore they may also be considered as potential targets for cancer therapy (28, 29).

Since HA and HP are popularly consumed as herbal teas (water extract) for the relief of digestive tract disorders, and they contain quercetin (mainly as glycosides), which we have shown in a previous study to possess anticarcinogenic activity against colon cancer cells (30), the antiproliferative and proapoptotic effects of the water extracts of these two *Hypericum* plants were tested in two human colon cancer-derived cell lines, HCT15 and CO115. These cell lines harbour different activating mutations that affect both MAPK/ERK and/or PI3K/Akt pathways: HCT15 has a KRAS (G13D) mutation (31) while CO115 harbour a BRAF (V599E) mutation (32), being representative of many CRC cases. The involvement of stress induced kinases p38 and JNK, and apoptotic markers were also studied.

Materials and Methods

Reagents and Antibodies

All reagents and chemicals used were of analytical grade. Wortmannin (W), LY-294,002 (LY), propidium iodide (PI), staurosporine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and chlorogenic acid (CA) were purchased from Sigma-Aldrich (St. Louis, MO, USA); PD-98059 (PD) was from Calbiochem (San Diego, CA, USA); zVAD-fmk was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Stocks solutions of W, LY, PD and zVAD were made in dimethyl sulfoxide (DMSO) and aliquots were kept at -20 ºC. DMSO (0.5%, final concentration) was used in the other conditions (control and HA extract alone) to exclude any solvent effect.

The primary antibodies, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-PTEN (Ser380/Thr382/383), anti-PTEN, anti-p44/42 MAPK and anti-phospho-p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling (Danvers, MA, USA); the anti-phospho-ERK, anti-Raf-B, anti-K-Ras, anti-PKC total, anti-PARP-1, anti-p38, anti-phospho-JNK and anti-JNK were from Santa Cruz Biotechnology, Inc.; the anti-caspase-3 was from Calbiochem (San Diego, CA, USA); and the anti-β-actin from Sigma-Aldrich. The secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were from GE Healthcare (Bucks, UK).

Cell culture and conditions

HCT15 and CO115 human colon carcinoma-derived cell lines were a gift from Dr. Raquel Seruca (IPATIMUP, University of Porto, Portugal) and were maintained in culture at 37 ºC in a humidified 5 % CO_2 atmosphere in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 mM HEPES, 0.1 mM pyruvate, 1 % antibiotic-

antimycotic solution (Sigma-Aldrich) and 6 % fetal bovine serum (FBS; EU standard, Cambrex, Verviers, Belgium). Cells were seeded onto six (2 ml) and twelve (1 ml) well plates at a density of 0.75×10^5 (HCT15) and 1.0×10^5 (CO115) cells/ml. Incubations for 48 h with different concentrations of the water extracts were performed for MTT, TUNEL and cell cycle analysis, and for 24 h and 48 h for western blot.

Preparation of HA extract

Plants of *H. androsaemum* were cultivated at Canidelo, Northern of Portugal, in a farm owned by Cantinho das Aromaticas Lda., whereas *H. perforatum* were obtained from Mapprod Lda., Braga, Portugal; plants are kept in active bank under the responsibility of the respective companies. The aerial parts of the plants were collected in July 2008 for HA and in July 2009 for HP; then, they were air-dried before being subjected to the water extraction by infusion. Batches of dried plant material are maintained at -20 ºC under the responsibility of CITAB with the accession numbers HA102008 and HP072009, for HA and HP, respectively. The plant infusions were prepared by pouring 150 ml of boiling deionized water onto 2 g of air-dried plant material and allowing it to steep for 5 min. After filtering, the water extracts were lyophilized to dryness and yields in terms of initial crude plant material dry weight of 27.0% (w/w) and 16.7% (w/w), for HA and HP, respectively, were obtained. Phenolic compounds were analyzed by HPLC as previously performed (12) and, for HA, a similar composition with a previous report of a water extract was obtained (15). The main phenolic compounds found in the plant water extracts differ in quantity between them. The following compounds are present. In HA: chlorogenic acid (CA) and isomer (3-*O* and 5-*O*-caffeoylquinic acid; 53.82 μg/mg), quercetin 3-galactoside (16.35 μg/mg), quercetin 3-glucoside (5.41 μg/mg), quercetin 3-rutinoside (2.73 μg/mg) and quercetin

(1.32 μg/mg). In HP: quercetin 3-rutinoside (38.07 μg/mg), quercetin 3-galactoside (16.00 μg/mg), quercetin 3-glucoside (6.47 μg/mg), chlorogenic acid (CA) and isomer (3-*O* and 5-*O*-caffeoylquinic acid; 5.75 μg/mg), quercetin (3.39 μg/mg), quercetin 3-rhamnoside (0.24 μg/mg), amenthoflavone (0.33 μg/mg) and hypericin (0.03 μg/mg). CA is much more abundant in HA water extract than in the HP water extract where quercetin and related compounds are the most representative (11).

Cell proliferation/viability assay

MTT reduction assay was used to estimate the number of viable cells after treatment with *Hypericum* water extracts, as previously described (30). Cells were treated with different concentrations of water extracts and CA for 46 h followed by two hours in the presence of MTT (final concentration 0.5 mg/ml). Hydrogen chloride 0.04 M in isopropanol was then used to dissolve the formazan crystals. The number of viable cells in each well was estimated by spectrophotometry. To discriminate between inhibition of cell proliferation (values between 0 and 100%) and induction of extensive cell death (negative values) the control values from the beginning of the treatment period (0 h) were subtracted from all samples collected after 48h of incubation, including the control. Results are presented as mean ± SEM of at least three independent experiments.

Assessment of apoptosis by TUNEL assay

Cells treated with different concentrations of HA (with or without 20 μM zVAD) for 48 h were collected (both floating and attached cells) and fixed with 4% paraformaldehyde for 15 min at room temperature and then attached into a polylysine treated slide using a Shandon Cytospin 4 (Thermo Scientific, Waltham, MA, USA).

Cells were washed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. TUNEL (TdT mediated dUTP Nick End Labelling) assay was performed using a kit from Roche (Mannheim, Germany), following the manufacturer's instructions. Cells were incubated with Hoechst for nuclei staining. The percentage of apoptotic cells was calculated from the ratio between TUNEL positive cells and total number of cells (nuclei staining with Hoechst), from a count higher than 500 cells per slide under a fluorescent microscope. Results are presented as mean ± SEM of at least three independent experiments.

Protein extraction and western blot

After incubation periods, cells were first washed with PBS and then lysed for 15 min at 4 ºC with ice-cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA), supplemented with 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM $Na_2V_3O_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with BSA as a protein standard. Twenty micrograms of protein from each sample were separated by SDS gel electrophoresis and then electroblotted to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) non-fat dry milk or BSA, incubated with the primary antibody followed by the secondary antibody conjugated with IgG horseradish peroxidase. Immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using

the Quantity One software from Bio-Rad. β-actin was used as a loading control. Results are presented as mean ± SEM of at least three independent experiments.

Cell Cycle analysis

Cells were treated with HA for 48 h and then harvested (both floating and attached cells) by tripsinization. After centrifugation, cell pellets were washed in PBS, fixed with ethanol 70% at 4ºC for 15 min and rinsed with PBS. Cells were then incubated with staining solution (50µg/ml propidium iodide and 20µg/ml RNase A in PBS) at 37ºC for 15 min. Cell cycle progression was analyzed by flow cytometry using a Coulter Epics XL Flow Cytometer (Beckman Coulter Inc., Miami, FL, USA) counting at least 40,000 single cells per sample. Phases of cell cycle were fitted using the mathematical Watson Pragmatic model (33) with the FlowJo Analysis Software (Tree Star, Inc., Ashland, OR, USA).

Statistical analysis

Student's t-test or one-way ANOVA followed by the Student-Newman-Keuls test was used to perform statistical analysis for TUNEL, cell cycle and western blot data. GraphPad Prism 4.0 software (San Diego, CA, USA) was used and *P*-values ≤ 0.05 were considered statistically significant.

Results

Effects of two *Hypericum* species on cell proliferation and apoptosis

To evaluate the anticancer potential of water extracts from *H. perforatum* and *H. androsaemum* their effects for 48h in the number of viable cells, in HCT15 and CO115

human colon carcinoma-derived cell lines, were investigated using the MTT assay. As shown in Figure 1A, HCT15 and CO115 cell lines are more resistant to HP water extract than to HA water extract. The number of viable cells decreased remarkably with HP treatment only at concentrations above 200 µg/ml in both cell lines. The HA extract was more efficient in inhibiting cell proliferation in a concentration-dependent manner in both cell lines, with an IC50 (the concentration that inhibited cell growth by 50%) of around 85 µg/ml in HCT15 and 65 µg/ml in CO115 cells, compared to HP. At the highest concentrations tested of HA, extensive cell death was observed in both cell lines, as indicated by both the presence of significant amounts of floating cells and by PI staining (data not shown). Incubation of CRC cells with HA also significantly induced apoptosis in a concentration-dependent manner in both cell lines (Figure 1B), as shown by the TUNEL assay. Comparing both *Hypericum* water extracts, HA has, therefore, higher anticancer potential as compared to HP.

In order to characterize effects of HA on signaling pathways related to proliferation and apoptosis, the IC50 concentration for each cell line (85 µg/ml for HCT15 and 65 µg/ml for CO115) and a concentration below this were used and their effects on the levels of relevant molecular targets characterized in subsequent experiments.

Effect of chlorogenic acid (CA) on cell proliferation

H. androsaemum water extract was more efficient in inhibiting cell growth in both HCT15 and CO115 CRC cell lines than HP. In an attempt to find the compound responsible for these effects, the main phenolic present in this extract, chlorogenic acid (CA), which also distinguishes this extract from HP water extract that has a much lower concentration, was evaluated individually at different concentrations, using MTT assay.

CA is present at about 54 μg/mg in HA water extract, while in HP CA is present in smaller amounts (about 6 μg/mg, respectively). No effects were observed on cell proliferation in neither of the cell lines treated with CA up to 200 μM, which corresponds to a concentration 20 times higher than the one found in 100 μg/ml of HA water extract (Figure 2). It seems therefore that the inhibition of cell proliferation produced by HA is not due to CA alone.

Effects of HA on PI3K/Akt and MAPK/ERK pathways

Constitutive activations of MAPK/ERK and PI3K/Akt pathways are present in a large number of CRC cases, leading to an increase of proliferation and an inhibition of apoptosis (20). Phosphorylation of ERK and Akt, respectively, are indicators of their activation. As shown in Figure 3A, HA significantly decreased phospho-Akt protein level in CO115 cells, in a concentration-dependent manner, after 24 h of incubation. This effect was not observed in HCT15 since there was no detectable amounts of phospho-Akt in these cells (data not shown), which is in agreement with previous observations (30). As expected, reference inhibitors of PI3K, wortmannin (W) and LY-294,002 (LY), also significantly decreased phospho-Akt levels (34). The effect of HA on phospho-PTEN, a negative regulator of PI3K/Akt pathway, was also tested. As shown in Figure 3B, HA did not change phospho-PTEN levels in HCT15. As previously reported (30), no detectable expression of PTEN was observed in CO115 cells (data not shown).

Concerning effects on the MAPK/ERK pathway, a significant decrease in phospho-ERK protein level was observed in CO115 cells, but not in HCT15, induced by the higher concentration of HA tested (Figure 3C). As expected, a significant reduction of phospho-ERK levels in both cell lines was also induced by PD-98059 (PD), a

reference inhibitor of the MAPK/ERK pathway (35). In addition, we also treated both cell lines with CA. This compound did not decrease phospho-Akt or phospho-ERK protein levels at 10 and 100 μM in both cell lines (data not shown).

Effects of HA on BRAF and KRAS levels

Subsequently, since KRAS activates both MAPK/ERK and PI3K/Akt pathways and BRAF activates MAPK/ERK pathway, effects of HA on the protein expression of KRAS and BRAF oncogenes were studied. As shown in Figure 4A, the higher HA concentration tested was able to significantly decrease the levels of BRAF in CO115 (cells with mutant BRAF). In HCT15 cells, which express the wild type BRAF, no effect of HA on BRAF protein expression was observed. No significant changes were observed in wild type or mutant KRAS levels induced by HA (Figure 4B). CA, when used individually, did not change KRAS or BRAF levels at 10 and 100 μM in both cell lines (data not shown).

Effects of HA on p38 and JNK pathways

The effect of HA on p38 and JNK signalling pathways, two stress-activated protein kinases that are involved in the control of proliferation and induction of apoptosis (27), were also studied. Our results showed a remarkable induction of phospho-p38 expression at both concentrations tested and of phospho-JNK expression mainly at the higher concentration tested after 48 h in both cell lines (Figure 5A and 5B).

Effects of HA on cell cycle and death mechanisms

As shown in Figure 1B with TUNEL assay, p53-mutated HCT15 cells are more resistant to apoptosis induction by HA than the p53-wild type CO115 cells. Corroborating these results, when cell cycle analysis was performed, the sub-G1 fraction of cells (indicative of DNA fragmentation typical of apoptosis) was considerably higher in CO115 as compared with HCT15, when treated with HA (Figure 6A, 6C).

In order to verify the role of caspase activation on the apoptotic effect of the HA water extract, caspase-3 and Poly (ADP-ribose) polymerase-1 (PARP-1) expressions by western blot were studied. As shown in Figure 5C, HA increased cleaved caspase-3 and cleaved PARP-1 in CO115 cells. In HCT15, we did not observe cleaved caspase-3 or cleaved PARP-1, although a decrease in total caspase-3 and PARP-1 were detected. A higher expression level of total PARP-1 was observed in HCT15 as compared to CO115. The cleavage of caspase-3 and PARP-1 were also induced by staurosporine, an apoptotic inducer used here as positive control, in both cell lines (data not shown).

In order to confirm the role of caspase activation on apoptosis induction by HA, cells were incubated in the presence of the general caspase inhibitor zVAD, and apoptotic cells analysed by the TUNEL assay. As shown in Figure 5D, zVAD was able to prevent apoptosis induction by HA in both cell lines.

The ability of HA to inhibit cell cycle was also confirmed by the cell cycle analysis. As shown in Figure 6A and 6B, HA induced both S phase and G2/M phase arrest in a concentration-dependent manner in both cell lines. The effect was more pronounced in the apoptosis-resistant HCT15 cell line.

Discussion

In the present study, the potential anticancer effects of water extracts of the medicinal plants *H. androsaemum* and *H. perforatum*, as well as, the main phenolic constituent present in HA extract, chlorogenic acid (CA), were studied in HCT15 and CO115, a KRAS and BRAF mutant human colorectal-derived cell lines. HA efficiently inhibited cell proliferation and induced apoptosis in a concentration-dependent manner in both cell lines. CO115 cells showed to be more sensitive to HA extract (IC50 ~65 μg/ml) when compared with HCT15 (IC50 ~85 μg/ml). As far as we know, this is the first report of the anticancer effect of *H. androsaemum* water extract in CRC, which is popularly used in Portugal to treat problems of the gastrointestinal tract. The extract HP did not show significant effect on cell growth in neither of the cell lines. Previously, we reported that quercetin has antiproliferative effects on these colorectal cancer cells (30), and since HP water extract is rich in quercetin and related compounds, this result was somewhat surprising. Anticarcinogenic activities have been found for *H. perforatum* in other cell types (4-7) being related with one of its main constituents, hypericin (8-10), which is present in HP water extract (although in very small amounts) but not in the water extract of HA (11, 36). These results suggest that the anticancer effects observed for HA reflect the presence of other compounds in this species. We, therefore, studied the antiproliferative effect of the CA, which is much more abundant in HA than HP. However, when used alone this compound did not have any effect on cell proliferation in neither of the cell lines, which indicates that the HA's effects are not due to its major phenolic compound, CA. Thus, other compounds or a synergism between the compounds present in the water extract of HA may be responsible for the HA effects.

The differences in the genetic background of the two cell lines used allowed the study of the relevance of KRAS mutation versus BRAF mutation for HA's effects. In CO115 (that harbour a BRAF mutation and overexpress Akt) a significant decrease of

phospho-Akt expression was observed in a HA concentration-dependent manner. These results show the ability of HA to decrease PI3K/Akt signalling probably by inhibiting PI3K activity, as also shown for some individual flavonoids, such as quercetin (30, 34, 37). HA was also able to decrease MAPK/ERK signalling in CO115, as shown by a significant decrease in the phospho-ERK expression levels. Importantly, HA also decreased BRAF expression in these cells. Since HA did not affect the expression of phospho-ERK or BRAF in HCT15, our results indicate that the HA water extract affects the MAPK pathway at the level of mutant BRAF (Figure 7), which was confirmed by a decrease in mutant BRAF cell proliferation (CO115) versus no effect on wild-type (wt) BRAF cells (HCT15) at 60 μg/ml. Previous studies have shown that pharmacologic inhibition of RAF is highly effective at inhibiting the growth of BRAF mutant CRC cells (38). Moreover, recent reports show that RAF inhibitors block MAPK signalling in tumor cells harbouring mutant BRAF but activate this pathway in cells harbouring wt BRAF, emphasizing the importance of inhibiting specifically mutant BRAF to avoid secondary effects (39, 40). CA, the main phenolic compound present in HA water extract, has, however, no effect on levels of phospho-Akt, phospho-ERK or BRAF in CO115 cells.

Regarding the RAS oncogene, no changes on the levels of KRAS were observed by HA. Previously, we showed that quercetin, at around 20μM, inhibits cell proliferation in association with a decrease in KRAS levels (30). Although the HA extract contains quercetin and glycosides of quercetin, their concentrations are low (less than 5μM), which may explain the lack of effect of HA on KRAS. The HA-induced inhibition of cell proliferation and increased apoptosis in HCT15 KRAS mutated cells seems not to result from effects on MAPK/ERK or PI3K/Akt signalling.

The effect of HA on the other two major MAPK pathways, the p38 and JNK, were also studied, since these stress-activated kinases may be involved in the control of proliferation and/or apoptosis (27, 41). HA significantly induced the phosphorylation of p38 and JNK in both cell lines (Figure 7). The effects on these two pathways could explain the decreased on cell proliferation and cell cycle arrest. An induction of caspase-3 and PARP-1 cleavage was also observed in CO115 when incubated with HA. The induction of JNK by HA may contribute to the activation of the mitochondrial caspase cascade (28) and lead to the high levels of caspase-dependent apoptosis observed in CO115. On other hand, cleavage of caspase-3 or PARP-1 was not observed in HCT15 despite the induction of JNK. That probably happened because the percentage of apoptosis induction by HA is low (about 5%) in HCT15 cells. Nevertheless, a decrease of total caspase 3 and PARP-1 protein levels, as well as prevention of apoptosis by the caspase inhibitor zVAD were observed, which indicates that apoptosis induction is also caspase-dependent in this cell line. The resistance to apoptosis of HCT15 cells as compared to CO115 cells was also shown previously (30, 42) and may be explained by its p53 mutation status (31, 43, 44). In the p53-mutated HCT15 cells S-phase and G2/M-phase cell cycle arrest were more pronounced than induction of apoptosis, whereas the contrary was observed in the p53-wild type CO115 cells.

Studies with CA in skin cancer have shown that this phenolic acid has an anti-inflammatory effect, interfering with NF-kβ activation and COX-2 activity, and has an inhibitory effect on skin cancer promotion (45, 46). However, studies in CRC agree with our results where no effect on colonic cell proliferation has been observed for CA (46, 47). In an attempt to identify the active compound(s) responsible for the HA effects' on BRAF and phospho-ERK levels, a fractionation of the HA water extract was

performed with ethanol and methanol. The soluble and insoluble fractions of HA extract on both methanol and ethanol (20 mg lyophilized water extract per ml of solvent) were tested in cells (at same concentrations as those in the IC50 concentration of the crude extract). None of the fractions were able to significantly affect BRAF or phospho-ERK expression (data not shown). It seems, therefore, that the effects of HA water extract are due to a synergism between compounds, which explain why after fractionation of the extract the effect is lost.

In conclusion, our study shows that the water extract of *H. androsaemum* inhibits cell proliferation and induces apoptosis in CRC-derived cell lines more efficiently than that of *H. perforatum*. Of particularly interest, the effects of HA involve a specific inhibition of mutant BRAF, which leads to an inhibition of MAPK/ERK pathway in BRAF mutant cells but not in wt BRAF cells. Inhibition of PI3K/Akt pathway, as well as, an induction of both p38 and JNK pathways may also contribute to the anticancer activity of HA. Chlorogenic acid, the main phenolic compound present in the HA extract, showed not to be responsible for the anticancer effects observed for the extract. These data add *H. androsaemum* to the list of plants with potential to be included in dietary strategies for the control of CRC progression, particularly for tumors presenting BRAF mutations.

Acknowledgements

CPRX was supported by the Foundation for Science and Technology (FCT), Portugal, through the grant SFRH/BD/27524/2006 and the work was supported by the FCT research grants PTDC/AGR-AAM/70418/2006 (HypericumBiotech) and PEst-C/BIA/UI4050/2011. All projects are co-funded by the program COMPETE from QREN with co-participation from the European Community fund FEDER.

Conflict of interest statement

The authors do not have conflicts of interest.

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Figure Legends

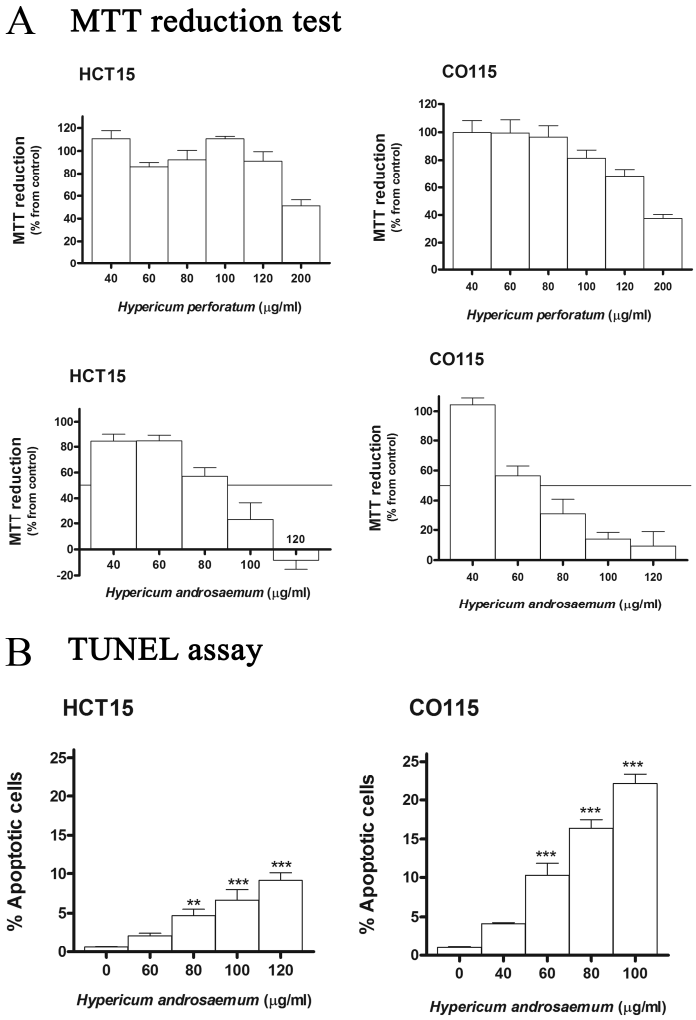


Figure 1. (**A**) Effects of different concentrations of water extracts of *Hypericum perforatum* and *Hypericum androsaemum* on cell viability/proliferation assessed by MTT reduction. (**B**) Effect of *Hypericum androsaemum* on apoptosis assessed by TUNEL assay, for 48 h, in HCT15 and CO115 cells. Values are mean ± SEM of at least 3 independent experiments. ** P≤ 0.01 and *** P≤ 0.001 when compared to control. In A: line represents the inhibition of 50% of cell proliferation (IC50); the negative value mean that the cells reduction capacity after 48 h in that condition was below than the one obtained in the control in the beginning of the treatment period (0 h), being a indirect indication of cell death by necrosis.

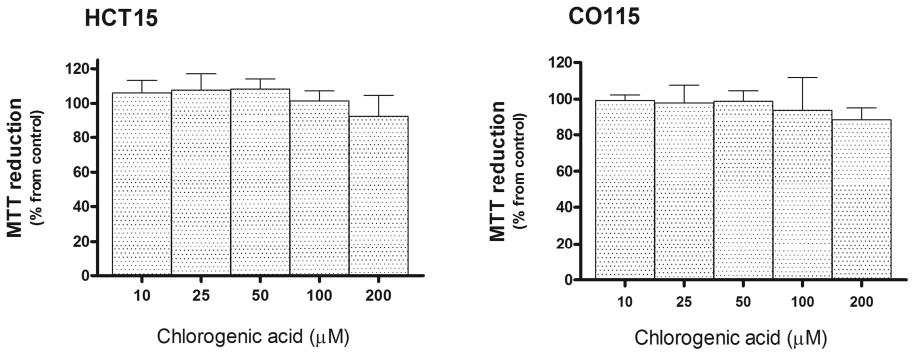


Figure 2. Effects of different concentrations of chlorogenic acid on cell viability/proliferation assessed by MTT reduction.

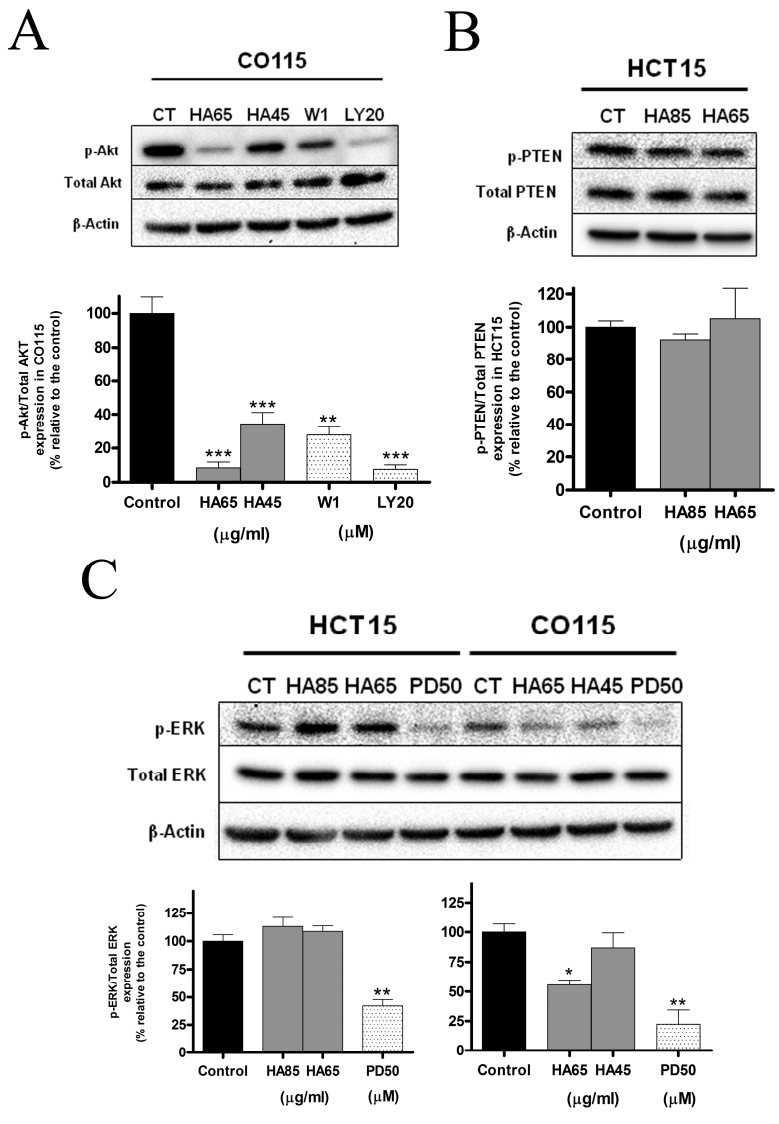


Figure 3. Effects of *Hypericum androsaemum* (HA) for 24 h on the levels of phospho-Akt in CO115 cells (**A**), phospho-PTEN in HCT15 cells (**B**) and phospho-ERK in

HCT15 and CO115 cells (**C**) at 85 µg/ml (HA85), 65 µg/ml (HA65) and 45 µg/ml (HA45), using western blot. β-Actin was used as loading control. Wortmannin 1 µM (W1) and LY-294,002 20 µM (LY20) were used as a reference inhibitor of PI3K and PD-98059 50 µM (PD50) was used as a reference inhibitor of phospho-ERK. Values are mean ± SEM of at least 3 independent experiments. * P≤ 0.05, ** P≤ 0.01 and *** P≤ 0.001 when compared to control.

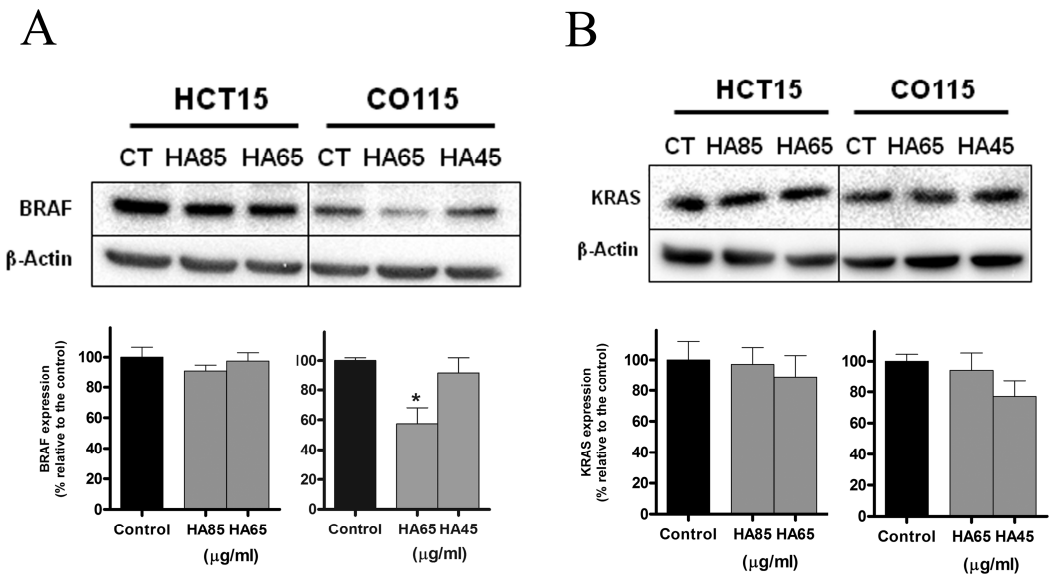


Figure 4. Effects of *Hypericum androsaemum* (HA) for 24 h on BRAF (**A**) and KRAS (**B**) levels in HCT15 and CO115 cells at 85 µg/ml (HA85), 65 µg/ml (HA65) and 45 µg/ml (HA45), using western blot. β-Actin was used as loading control. Values are mean ± SEM of at least 3 independent experiments. * P≤ 0.05 when compared to control.

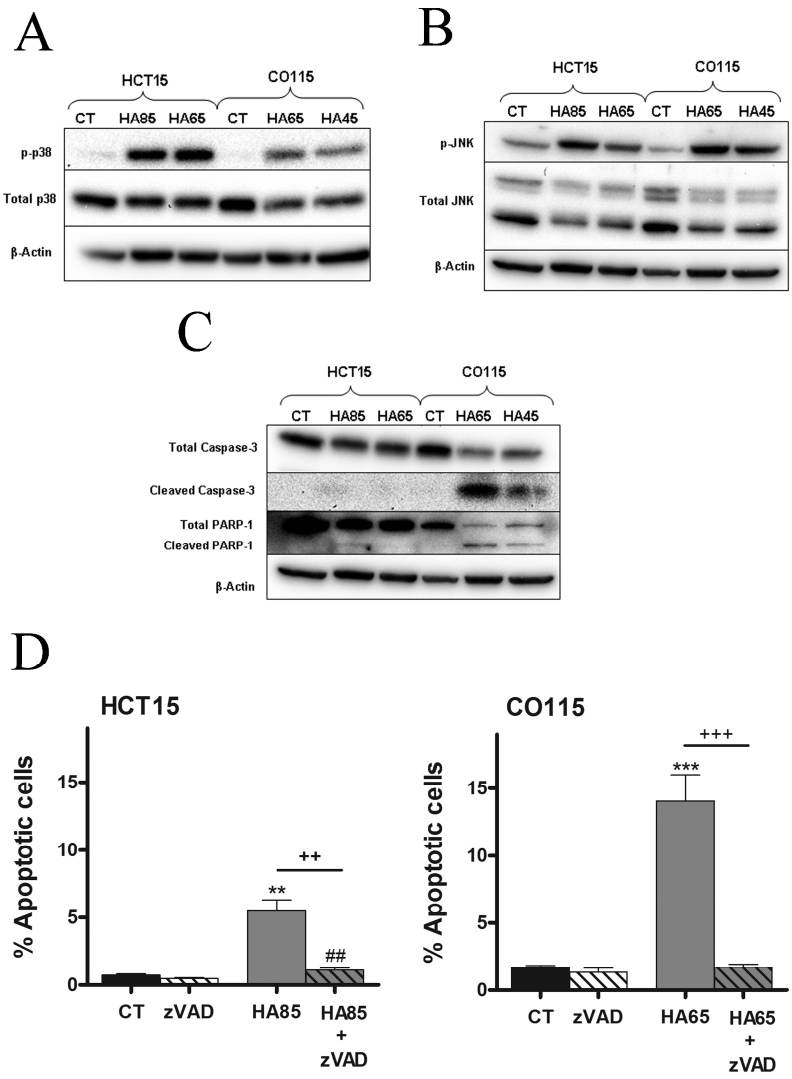
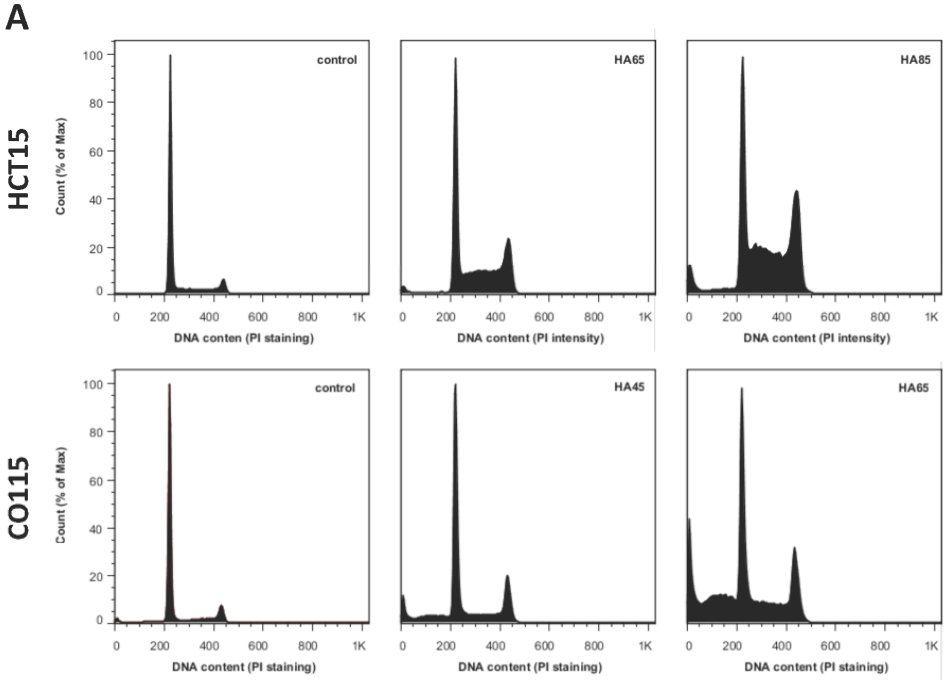


Figure 5. Effects of *Hypericum androsaemum* (HA) for 48 h on the levels of phospho-p38 (**A**), phospho-JNK (**B**) and apoptosis (**C, D**) in HCT15 and CO115 cells at 85 µg/ml (HA85), 65 µg/ml (HA65) and 45 µg/ml (HA45). In **A**, **B** and **C**, levels of proteins were studied by western bloting. β-Actin was used as loading control. Images are representative of at least 3 independent experiments. In **D**, the effect of HA alone or in combination with 20 µM z-VAD-fmk (zVAD) on apoptosis was measured by the TUNEL assay. Values are mean ± SEM of at least 3 independent experiments. ** $P \leq 0.01$ and *** $P \leq 0.001$ when compared to control (CT); $^{\#\#}$ $P \leq 0.01$ when compared to zVAD alone; $^{++}$ $P \leq 0.01$ and $^{+++}$ $P \leq 0.001$ between each other.

A



B

		Cell cycle phase (% of cells)		
	Treatment	G1/G0	S	G2/M
HCT15	control	60.8 ± 4.1	28.8 ± 0.8	10.4 ± 3.9
	HA65	36.0 ± 2.1***	47.9 ± 3.1***	16.1 ± 2.5
	HA85	28.7 ± 3.1***	51.2 ± 1.4***	20.1 ± 4.4*
CO115	control	60.5 ± 9.6	28.1 ± 6.5	11.4 ± 5.3
	HA45	55.2 ± 3.9	27.1 ± 1.1	17.7 ± 4.2
	HA65	40.1 ± 1.7*	35.7 ± 1.4	24.2 ± 2.8*

C

Sub-G1 (%)	Treatment			
Cells	control	HA45	HA65	HA85
HCT15	2.6 ± 1.2	---	4.1 ± 1.1	8.0 ± 0.9**
CO115	4.0 ± 2.0	12.7 ± 4.4*	30.4 ± 5.1**	---

Figure 6. Effects of *Hypericum androsaemum* (HA) for 48 h on cell cycle progression in HCT15 and CO115 cells at 45 µg/ml (HA45), 65 µg/ml (HA65) and 85 µg/ml (HA85) assessed by flow cytometry. (**A**) Distribution of single cells through the phases of cell cycle of a representative experiment. The effects of HA on cell cycle progression (**B**) and in the percentage of sub-G1 fraction (**C**) of 3 independent experiments are

shown. Values are mean ± SEM. * P≤ 0.05, ** P≤ 0.01 and *** P≤ 0.001 when compared to control.

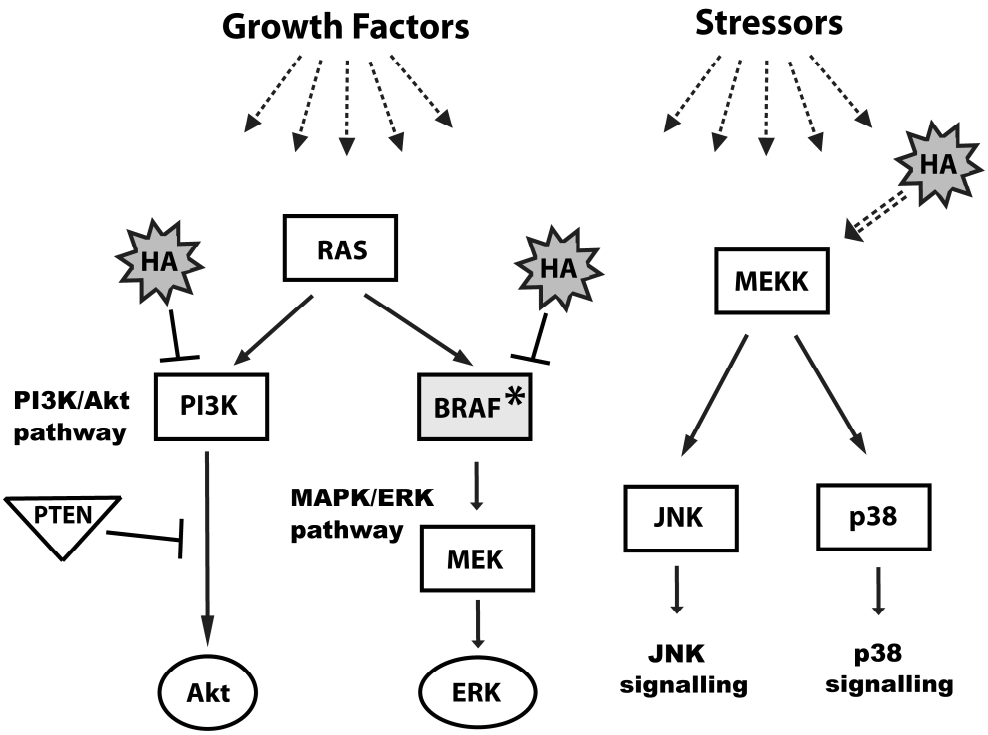
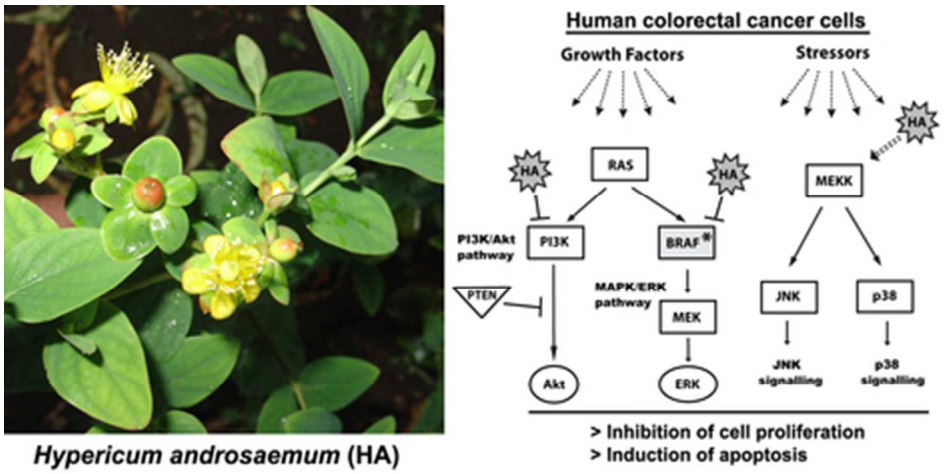


Figure 7. Proposed model for the inhibition of cell proliferation and induction of apoptosis in colon cancer cells by *Hypericum androsaemum* (HA), in particular with effects on PI3K/Akt, MAPK/ERK, JNK and p38 signaling pathways. The anticancer effect of HA could be due to an inhibition of PI3K/Akt pathway, a decrease on BRAF mutation leading to an inhibition of MAPK/ERK pathway and an induction of both p38 and JNK signalling.



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Hypericum androsaemum water extract inhibits proliferation in human colorectal cancer cells through effects on MAP kinases and PI3K/Akt pathway

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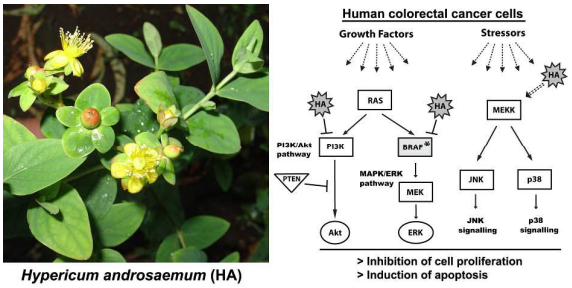
→ *Hypericum androsaemum* water extract has anticancer potential inhibiting MAP kinase pathway through effects on mutant BRAF.



***Hypericum androsaemum* (HA)**