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Insights on the antitumor effects of kahweol on human breast cancer: Decreased survival and increased production of reactive oxygen species and cytotoxicity

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ABSTRACT

The present study aims to identify the modulatory effects of kahweol, an antioxidant diterpene present in coffee beans, on a panel of human tumor cell lines. Kahweol inhibits tumor cell proliferation and clonogenicity and induces apoptosis in several kinds of human tumor cells. In the estrogen receptor-negative MDA-MB231 human breast cancer, the mentioned effects are accompanied by caspases 3/7 and 9 activation and cytochrome c release. On the other hand, kahweol increases the production of reactive oxygen species and their cytotoxicity in human breast cancer cells but not in normal cells. Taken together, our data suggest that kahweol is an antitumor compound with inhibitory effects on tumor cell growth and survival, especially against MDA-MB231 breast cancer cells.

1. Introduction

After water, coffee is – along with tea – the most commonly consumed drink in the world. Coffee is made from ground, roasted coffee beans (the dried seeds of coffee plant – *Coffea* – berries). Epidemiological studies associate the consumption of unfiltered coffee with a low incidence of colon and liver cancer [1,2]. A recent meta-analysis of cohort studies reinforces the suggestion that coffee consumption may reduce total cancer incidence [3]. Furthermore, a big prospective analysis concluded that there is a strong inverse association between coffee consumption and risk of lethal prostate cancer [4]. In a recent report, it has been shown that a high daily intake of coffee is associated with a statistically significant decrease in estrogen receptor-negative breast cancer among postmenopausal women [5]. Furthermore, its preventive effects against oxidative stress and DNA damage are well described [6]. Due to its high rate of consumption, coffee is a major source of some antioxidants in the Western diet. Coffee beans contain more than a thousand of compounds [7]. Kahweol (Fig. 1A) is an antioxidant diterpene of coffee beans that is more abundant in unfiltered coffee beverages, such as Turkish and Scandinavian coffee [8], with approximate kahweol contents of 5.4 and 7.2 mg per cup, respectively [9].

In many cases, the biological effects of kahweol have been studied in combination with cafestol, a closely related compound (in fact, kahweol is 1,2-didehydrocafestol). Assays performed with either pure cafestol or a cafestol–kahweol mixture indicated that kahweol contributes specially to increase serum concentration of alanine aminotransferase and has little additional effects on serum concentration of cholesterol, but reduces that of γ-glutamyltransferase [10,11]. In contrast, subsequent studies found out that kahweol really reduces γ-glutamyltransferase but concluded that it is unlikely that kahweol is the component of coffee oil that is responsible for the other liver enzyme level increases [12]. Evidence has accumulated showing anti-oxidant, anti-inflammatory, anti-tumoral and chemoprotective effects of kahweol. As an anti-oxidant, kahweol has been shown to protect against hydrogen peroxide induced oxidative stress and DNA damage, probably via scavenging reactive oxygen species (ROS) [6] and to induce heme oxygenase-1 to control intracellular ROS levels [13]. The anti-inflammatory action of kahweol includes its ability to suppress macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression [14,15] and its modulatory action on NF-kappaB pathway [16]. Our group has shown that kahweol behaves as an anti-angiogenic compound and targets several key inflammatory

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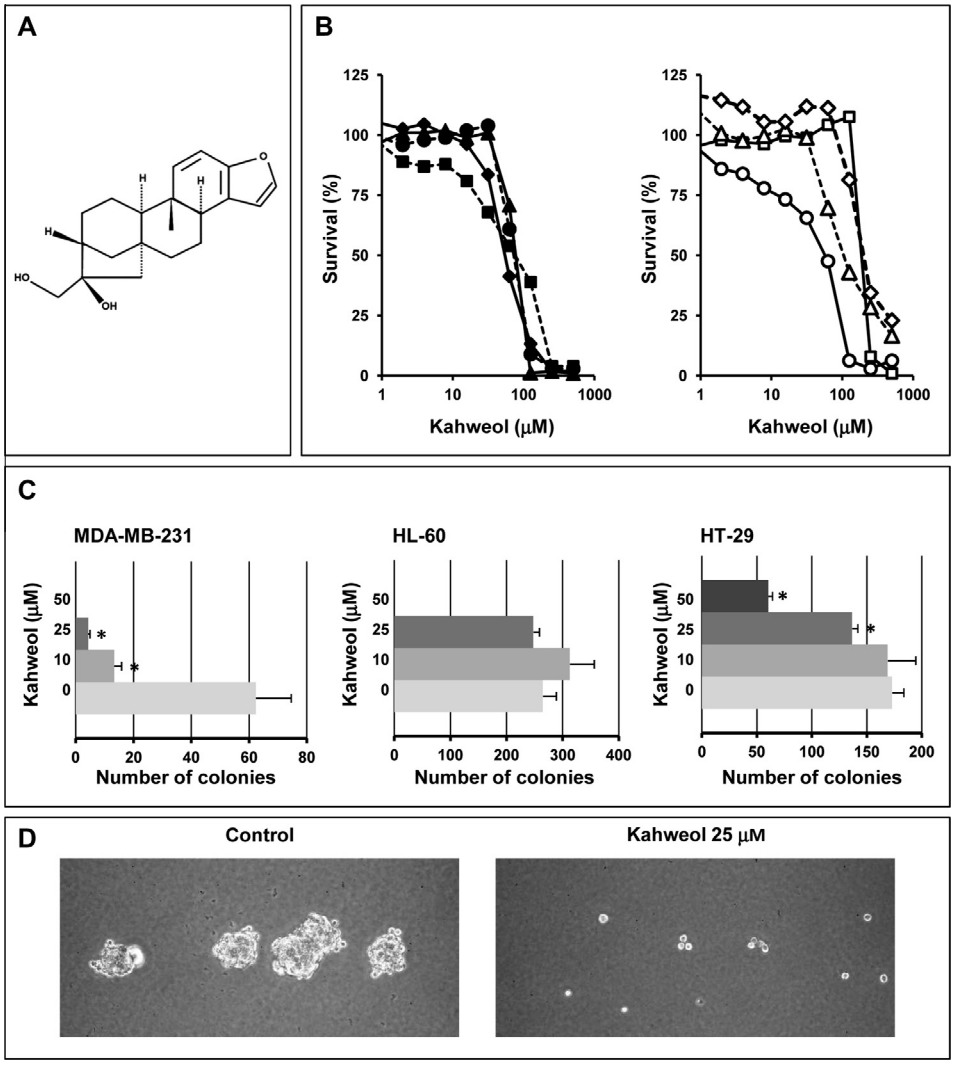

Fig. 1. (A) Chemical structure of kahweol. (B) Kahweol inhibits proliferation of cell lines. Survival curves of tumor cell lines treated with kahweol. Left: HL-60 leukemia (circles), HepG2 hepatoma (squares), HT-29 colon adenocarcinoma (rhombuses), and HT-1080 fibrosarcoma cells (triangles); right: estrogen receptor-negative MDA-MB-231 (circles), estrogen receptor-positive ZR-75-1 (squares) and MCF-7 (triangles) breast carcinoma cells, and human gingival fibroblasts (rhombuses). Concentrations are represented in logarithmic scale. Depicted data are means of values of three independent experiments, each one with quadruplicate samples. (C) Kahweol inhibits the clonogenicity of tumor cells. Histograms show the number of colonies after treatment of human MDA-MB231, HL-60 and HT-29 tumor cells with different concentrations of kahweol given as means ± S.D. of three different experiments. *Statistically significant ($p < 0.01$) as compared to control values, according to a two-tailed Student's *t*-test. (D) The most potent inhibitory effect is observed on MDA-MB-231 cells. The image shows untreated (control) and 25 μM kahweol-treated MDA-MB-231 cell colonies after two weeks of incubation. The image was taken using an inverted microscope under phase contrast at 100× magnification.

biomolecules [17]. Anti-tumor, pro-apoptotic effects of kahweol have been reported for several kinds of cancer cells. Kahweol-induced apoptosis is mediated by STAT3 phosphorylation inhibition in human lung adenocarcinoma A549 cells [18], down-regulation of AKT phosphorylation in human leukemia U937 cells [19], and modulation of the protein expression levels of SP1 regulatory genes in human malignant pleural mesothelioma [20]. Finally, kahweol exhibits Nrf2-mediated chemoprotective effects [21] inducing glutathione S-transferase, γ-glutamylcysteine synthetase, glucoronosyl transferase and O6-methylguanine-DNA methyltransferase [22–24].

Up to date, there is no report comparing kahweol effects on an array of different tumor cells. To contribute to fill this gap is a major aim of the present work. Our results show that, indeed, kahweol is able to inhibit the growth of several human cancer cell types, decreasing their proliferative rates and clonogenicity and inducing their apoptosis. Our data also indicate that the anti-tumoral effect is particularly relevant for the estrogen receptor-negative MDA-MB231 cell line, inducing an activation of the intrinsic pathway of apoptosis. On the other hand, we show that kahweol increases the production of reactive oxygen species and their cytotoxicity in human breast cancer cells but not in normal cells.

2. Materials and methods

2.1. Cell cultures and treatments

Transformed human HL-60 leukemia, HT-29 colon adenocarcinoma, HT-1080 fibrosarcoma, HepG2 hepatoma, and estrogen-receptor positive ZR75-1 and MCF-7 and estrogen receptor-negative MDA-MB-231 breast carcinoma cells were supplied by ATCC and maintained in culture as described by the provider. Human gingival fibroblast primary cultures were maintained in high glucose DMEM and cells in passage 5 were used for the experiments. All culture media were supplemented with 10% FBS and antibiotics. Kahweol was supplied by Santa Cruz Biotechnology,

Inc. (Santa Cruz, CA). Stock solution (10 mg/mL) was prepared in DMSO and stored in aliquots at −20 °C. In all the assays, the vehicle (DMSO) was at less than 1% (v/v) and controls with the vehicle alone were carried out in parallel.

2.2. MTT and soft-agar clonogenic assays

The MTT dye reduction assay in 96-well microplates was used as previously described [17]. All determinations were carried out in quadruplicate. IC_{50} value was calculated as the concentration of kahweol yielding a 50% of cell survival.

For the clonogenic assay, 6-well plates were used. Each well was covered with 1.5 mL of 0.5% (w/v) low melting agarose in complete culture medium and allowed to solidify. Over this agar layer, 1.5 mL of 0.35% (w/v) low melting agarose in complete culture medium containing 5×10^3 tumor cells was added and allowed to solidify. Both base and top agar were prepared in the absence or presence of different concentrations of kahweol. All wells were done in triplicate. After two weeks of incubation, cell colonies were stained with 0.005% (w/v) violet crystal (0.5 mL per well) for 1 h and colonies with a diameter greater than 0.1 mm were counted.

2.3. Cell cycle cytometric and other apoptosis assays

Hoechst staining and cell cycle cytometric procedures have been described by us elsewhere [25]. Caspase activity was determined as previously described [25]. Cytochrome c release assay was carried out as described by us elsewhere [26].

2.4. H_2O_2 cytotoxicity and production

To analyze the effect of kahweol pretreatment on H_2O_2 cytotoxicity, cells were treated with kahweol at the different tested concentrations for 8 h. Afterwards, the culture medium was changed, 200 μM H_2O_2 was added and cells were incubated for another 12 h. After washing, new medium was added and the MTT assay was carried out to evaluate cell survival.

To quantify H_2O_2 production by cell cultures, the ROS-Glo H_2O_2 assay kit (Promega) was used. Cells were seeded at 20,000 cells/well in opaque white wall plates, complete culture medium was substituted by medium without serum and with the different tested concentrations of kahweol. After 2 h of incubation, the provided substrate reagent was added. After another 6 h of incubation, the provided detection reagent was added. Finally, after 20 min of incubation luminescence was measured.

2.5. Statistics and image analysis

Quantitative data are expressed as means ± standard deviation (S.D.). Two-tailed Student's *t*-test was used for evaluations of pair of means, to establish which groups differed from the control group. Quantitative analysis of images was performed with the Scion Image and ImageJ Programs.

3. Results

3.1. Kahweol inhibits tumor cell proliferation

Since sustained proliferation is one of the hallmarks of cancer, we studied the effects of kahweol on the growth of several human tumor cell lines. Fig. 1B depicts survival curves obtained with the MTT assay. The IC_{50} values for kahweol treatment obtained from this assay show a potent inhibitory effect of kahweol in HT-1080 fibrosarcoma (82 ± 1 μM), HT-29 colon adenocarcinoma (61 ± 17 μM), HL-60 leukemia (75 ± 7 μM), HepG2 hepatoma (100 ± 25 μM), and estrogen receptor-positive ZR75–1 (198 ± 1 μM) and MCF-7 (109 ± 6 μM) and estrogen receptor-negative MDA-MB-231 breast carcinoma cells (60 ± 7 μM).The IC_{50} value for kahweol treatment of normal human gingival fibroblast was the highest (209 ± 5 μM).

3.2. Kahweol shows a potent inhibitory effect on MDA-MB-231 human breast cancer cells in the soft-agar clonogenic assay

Cell attachment to extracellular matrix (ECM) components uses to be required for cell proliferation. However, most cancer cells can proliferate independently of attachment to ECM. The soft agar clonogenic assay can be used to test this ability. Fig. 1C shows that kahweol inhibits in a dose-dependent manner the clonogenic potential of HT-29, HL-60, and MDA-MB-231 cells. Noteworthy, the most potent effect was observed on MDA-MB231 breast carcinoma cells, where even the lowest tested kahweol concentration (10 μM) produced an inhibitory effect higher than 80%. Furthermore, kahweol not only decreases the number of colonies but all their size (Fig. 1D).

3.3. Kahweol exhibits a strong pro-apoptotic effect on MDA-MB231 human breast cancer cells

Cell growth is the result of the balance between their proliferation and death rates. Therefore, it would be advisable to test the potential effects of kahweol on tumor cell apoptosis. Fig. 2A shows that a treatment with 25 μM kahweol was able to induce apoptosis in HT-29, HL-60 and MDA-MB231 tumor cells, but not in HT-1080 tumor cells. Fig. 2B shows greater details of a set of apoptotic MDA-MB231 cells upon 25 μM kahweol treatment.

An easy, quantitative way to follow the effects of a test compound on apoptosis is to assess the increase in sub-G1 cell population after treatment using a flow cytometric cell cycle analysis assay after propidium iodide staining. Fig. 2A shows that, in fact, 50 μM kahweol is able to increase sub-G1 MDA-MB231 human breast cancer cell population, which is not the case for the other three tumor cell lines tested. Fig. 2C shows that kahweol induces a dose-dependent increase of the sub-G1 population of MDA-MB231 cells and that this increase is accompanied by a kahweol dose-dependent decrease in the percentage of cells in G2/M phase.

We also tested the effects of kahweol treatment on caspases 3/7, and 9 activities. Fig. 3A shows that, in fact, kahweol treatment (in the tested range of concentrations, 10–50 μM) for 6 h was able to induce up to 3-fold caspase 3/7 activity. This is an activation similar to that induced by the positive control 2-methoxyestradiol. Fig. 3B shows time-course assays for caspases 3/7 and 9. Kahweol induced activation of both caspases activities. Fig. 3C shows that there was a dose-dependent increase in cytochrome c release to cytoplasm after 14 h of treatment.

3.4. Kahweol does not protect against hydrogen peroxide cytotoxicity and increases its production by human breast cancer cells

Since kahweol has been described as an anti-oxidant compound and nonetheless it compromises breast cancer cell survival, we next tested what effect had kahweol pretreatment on H_2O_2 cytotoxicity. Fig. 4A shows that kahweol increases H_2O_2 cytotoxicity in a dose–response manner in MCF-7 and even more in MDA-MB231 breast cancer cell but in contrast it has no effect in normal human gingival fibroblasts.

On the other hand, Fig. 4B shows that kahweol increases the production of H_2O_2 in both MCF-7 and MDA-MB231 breast cancer cells but not in normal human gingival fibroblasts.

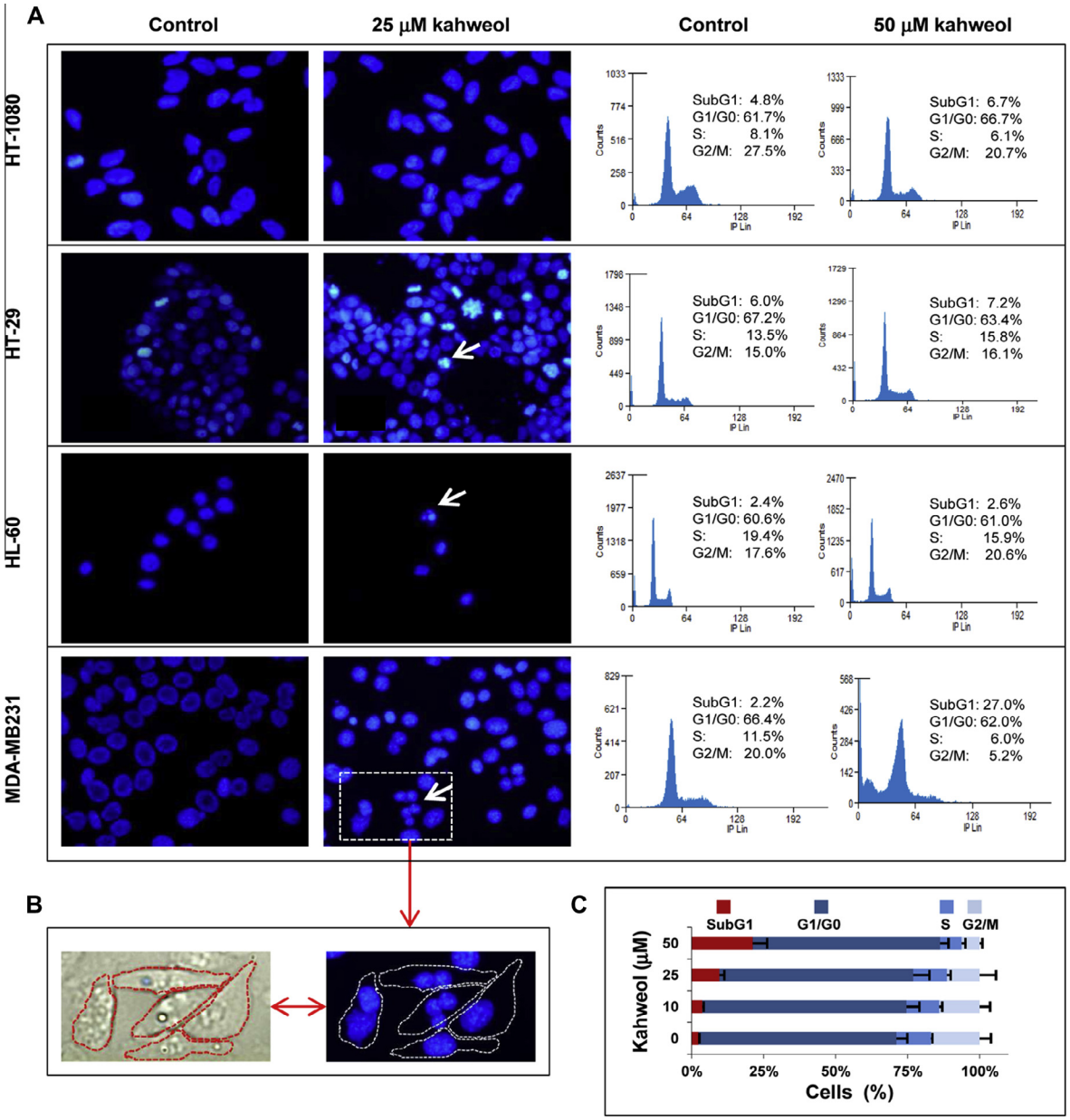

Fig. 2. Kahweol induces estrogen receptor-negative MDA-MB-231 breast carcinoma cell-specific apoptosis. (A) Although Hoechst staining shows evidence of several apoptotic nuclei in HT-29, HL-60 and MDA-MB-231 cells (photographs in the two left columns), flow cytometric analysis of cell cycle shows that kahweol only increases very significantly the percentage of subG1 subpopulation in MDA-MB-231 cells (two right columns). (B) A zoom of several apoptotic MDA-MB-231 cells using clear field image (left) and Hoechst staining (right). (C) Relative distribution of MDA-MB-231 cells in the different phases of the cell cycle at different concentrations of kahweol. Data are given as percentages of the total population and they are means ± S.D. of three independent experiments. *Statistically significant ($p < 0.01$) as compared to control values, according to a two-tailed Student's *t*-test.

4. Discussion

Coffee contains various antioxidants and phenolic compounds, some of which have antitumor effects in laboratory conditions [27]. Frequent coffee consumption is associated with diminished incidence of certain types of cancer [1–5]. One of the bioactive compounds related with the antitumor effects of coffee is kahweol (Fig. 1A) [18–20], a diterpene for which anti-oxidant and anti-inflammatory properties have also been reported previously [6,13–16]. Recently, we have described the anti-angiogenic effects of kahweol [17]. In the present work, different cells were treated with different concentrations of kahweol were used in a number of assays to test the potential effects (and selectivity) of this compound on tumor cell proliferation and survival.

The results obtained with the MMT assay (Fig. 1B) show that kahweol is an inhibitor of tumor cell proliferation in the micromolar concentration range. The lowest IC_{50} value (60 ± 7 μM) was observed for estrogen-receptor negative MDA-MB-231 breast carcinoma cells. In contrast, estrogen receptor-positive MCF-7 and ZR-75 breast cancer cells exhibited 2–3-fold higher IC_{50} values. These results are consistent with the previously published significantly higher breast cancer risk reduction associated with high coffee consumption for estrogen receptor negative compared to estrogen receptor positive tumors [5]. However, these differences are not great and the IC_{50} values obtained for the seven tested human tumor cell lines were also similar to that shown for normal human gingival fibroblasts and to that previously shown for endothelial cells [17]. For the rest of the study, we discarded tumor cell lines with IC_{50} values $\geqslant$125 μM.

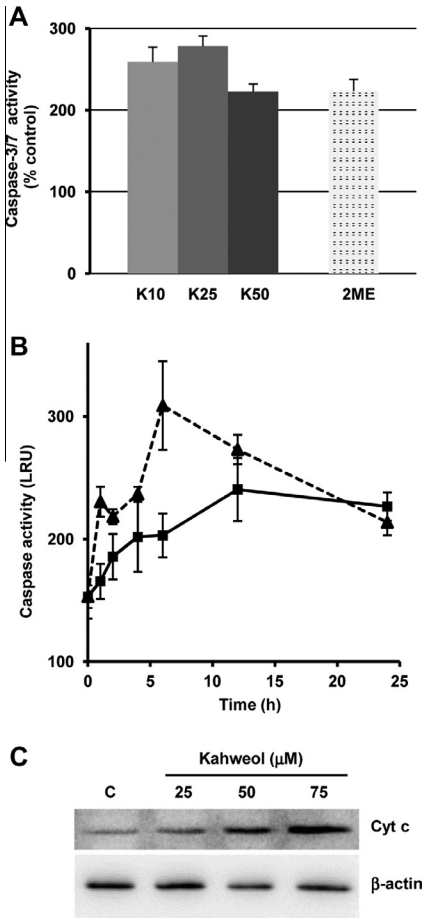

Fig. 3. Kahweol induces MDA-MB-231 cell apoptosis through the intrinsic pathway. (A) Activating effects of different concentrations of kahweol (K10, K25, K50, represent 10, 25 and 50 μM kahweol, respectively) on MDA-MB-231 cell caspase 3/7 activity after 6 h of treatment. Data for 20 μM 2-methoxyestradiol (2ME) treatment as a positive control are also shown. (B) Time course of the activating effects of 25 mM kahweol on caspase 3/7 (triangles) and caspase 9 (squares) activities. Data are given as percentages of caspase 3/7 activity (A) or as LRU units of caspase activity (B) and they are means ± S.D. of three different experiments. (C) Kahweol treatment induces cytochrome c release to MDA-MB-231 cell cytosol in a dose-dependent manner. Images from a representative Western blot assay are shown. Three independent experiments were carried out with similar results.

The results of the clonogenic assay on soft agar show differential effects suggesting certain cell specificity. HT-1080 fibrosarcoma cells were not able to form any colony on soft agar. Treatment significantly decreased the number of HT-29 colon adenocarcinoma and HL-60 leukemia cell colonies (as compared with control, non-treated cells) at concentrations equal or higher than 25 μM kahweol (Fig. 1C). Only in the case of estrogen receptor-negative MDA-MB-231 breast carcinoma cells a clear dose-effect was observed, with significant decreases in the number of colonies at $\geqslant$10 μM kahweol and a complete inhibition at 50 μM kahweol (Fig. 1C). Furthermore, for MDA-MB-231 cells, kahweol treatment did induce not only a decrease in the number of colonies but also in their size (Fig. 1D).

Since de-regulated proliferation and avoidance of apoptosis are two key hallmarks of cancer [28], we next studied the effects of kahweol on tumor cell apoptosis. Hoechst staining of cell nuclei after 24 h of treatment in the presence of 25 μM kahweol revealed (Fig. 2A, left, and Fig. 2B for a detailed view of apoptotic, kahweol-treated MDA-MB-231 cells) that some HT-29, HL-60 and MDA-MB-231 cells (but not HT-1080 cells) had entered apoptosis. Cell cycle analysis after 24 h of treatment in the presence of 50 μM kahweol allowed for the quantification of cell subpopulations within each cell cycle phase (Fig. 2A, right). Only in the case of MDA-MB-231 cells there was a great increase in the percentage of cells in sub-G1 (from 2.2% in control cells to 27.0% in kahweol-treated cells). This was concomitant to a similar decrease in the percentage of cells in the G2/M phase (from 20.0% to 5.2%), and a decrease in the percentage of cells in S phase (from 11.5% to 6.0%). This huge, 12-fold increase in the number of apoptotic MDA-MB-231 cells indicates a very potent pro-apoptotic effect of kahweol on these cells, as well as a remarkable selectivity and tumor type-specificity of this effect. Nonetheless, pro-apoptotic effects of kahweol have been previously reported for some other tumor cell types, including U937 human promonocytic leukemia cells, human lung adenocarcinoma A549 cells and human malignant pleural mesothelioma [18–20]. Among non-tumoral cells, we have shown anti-angiogenic effects of kahweol without apoptosis in HUVEC [17]. We expanded our analysis of cell cycle to treatments of MDA-MB-231 cells with different concentrations of kahweol. Our results (Fig. 2C) show a dose–response effect with increasing percentages of sub-G1 cell populations and concomitant decreasing percentages of G2/M cells.

In order to get mechanistic insights of the induction of MDA-MB-231 apoptosis by kahweol, we carried out dose–response and time-course assays of caspase activity. Results shown in Fig. 3 (A and B) confirm that kahweol is able to induce caspases 3/7, and 9 activities up to 3-fold, with maximum activation values after 6–12 h of treatment. These results are consistent with an activating role of kahweol on the intrinsic pathways of apoptosis. Dose–response increases of cytochrome c in the cytosol (Fig. 3C) reinforce the suggestion that kahweol can activate MDA-MB-231 cell apoptosis through the intrinsic pathway. This is in agreement with previously reported results in U937 human promonocytic cells [19].

Since kahweol has been described as an anti-oxidant agent, a question might arise concerning the congruence of the cytoprotective effects expected from an antioxidant and the claimed antitumor effects of kahweol. Although scientific literature shows overwhelming evidence in support of the antitumor effects of many anti-oxidant compounds, we wanted to respond to this concern focussing our attention on the effects of kahweol on H_2O_2 cytotoxicity and production. Our results show that H_2O_2 decreases estrogen receptor-negative MDA-MB231 cancer cell survival by more than 40% but has no effect on estrogen receptor-positive MCF-7 breast cancer cell and normal human gingival fibroblasts (Fig. 4A). Preincubation with increased concentrations of kahweol enhances H_2O_2 cytotoxicity in both breast cancer cell lines but has no significant effect on human gingival fibroblasts (Fig. 4A). Furthermore, kahweol enhances the production of H_2O_2 in both tested breast cancer cell lines but not in human gingival fibroblasts (Fig. 4B). Taken together, these results are consistent with the rest of results in this article, showing clear antitumor effects of kahweol on breast cancer cells.

A question might arise on how realistic or "physiological" are the doses we have examined. The scarce knowledge about the pharmacokinetics of kahweol in humans makes difficult to extrapolate laboratory concentrations to those achievable through oral administration. Nevertheless the doses used here are within the concentration range (from 10 to 100 μM) used by other authors.

In conclusion, our results show that kahweol has anti-proliferative and both potent anti-clonogenic and pro-apoptotic effects on MDA-MB-231. Additional experimental work seems warranted in order to increase our knowledge of the molecular mechanisms of the potent antitumor effects of kahweol on estrogen receptor-negative MDA-MB-231 breast carcinoma cells. The use of "omics" technologies and systems biology approaches [29] will contribute

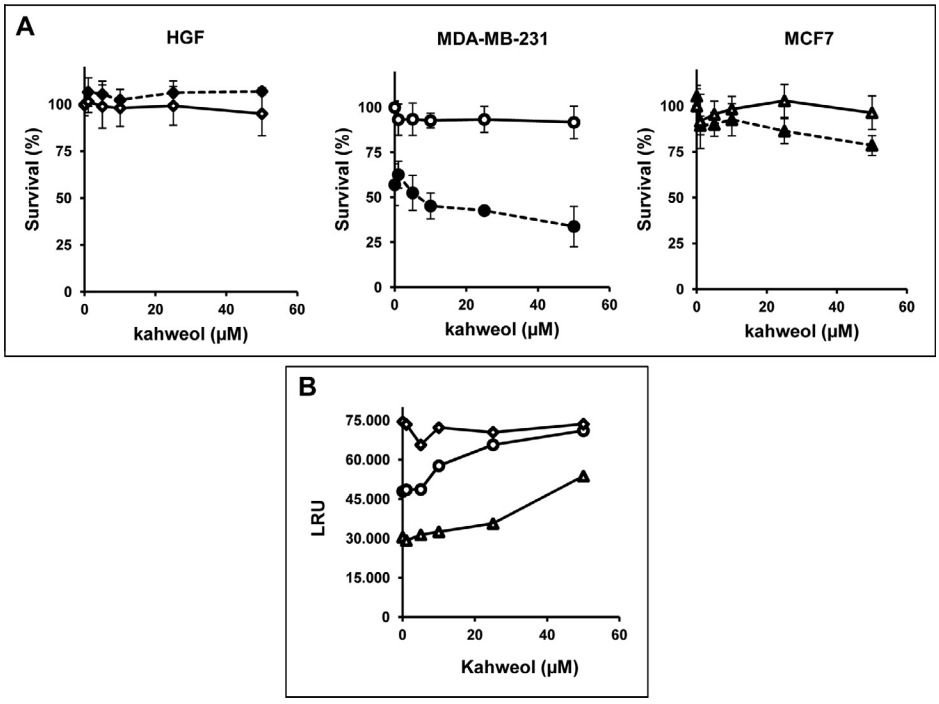

Fig. 4. Kahweol increases H_2O_2 cytotoxicity (A) and production (B) in human breast cancer cells but not in normal human gingival fibroblasts. (A) Results in the absence (open circles) and presence (circles) of 200 μM H_2O_2 are depicted. Data are given as percentages of cell survival and they are means ± S.D. of three different experiments. (B) Data are given for MDA-MB231 (circles) and MCF-7 (triangles) breast cancer cells and for human gingival fibroblasts (squares). Data are given as luminiscence relative units (LRU) and they are means of two different experiments.

to figure out a global view of the complex and interconnected regulatory and signaling networks involved in these effects.

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