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Research Paper

Treatment of liver cancer cells with ethyl acetate extract of *Crithmum maritimum* permits reducing sorafenib dose and toxicity maintaining its efficacy

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Abstract

Objectives Hepatocellular carcinoma (HCC) is one of the most frequent tumours worldwide and available drugs are inadequate for therapeutic results and tolerability. Hence, novel effective therapeutic tools with fewer side effects are of paramount importance. We have previously shown that *Crithmum maritimum* ethyl acetate extract exerts a cytostatic effect in HCC cells. Here, we tested whether *C. maritimum* ethyl acetate extract in combination with half sorafenib IC50 dose ameliorated efficacy and toxicity of sorafenib in inhibiting liver cancer cell growth. Moreover, we investigated the mechanisms involved.

Methods Two HCC cell lines (Huh7 and HepG2) were treated with *C. maritimum* ethyl acetate extract and half IC50 sorafenib dose usually employed *in vitro*. Then, cell proliferation, growth kinetics and cell toxicity were analysed together with an investigation of the cellular mechanisms involved, focusing on cell cycle regulation and apoptosis.

Key findings Results show that combined treatment with *C. maritimum* ethyl acetate extract and half IC50 sorafenib dose decreased cell proliferation comparably to full-dose sorafenib without increasing cell toxicity as confirmed by the effect on cell cycle regulation and apoptosis.

Conclusions These results provide scientific support for the possibility of an effective integrative therapeutic approach for HCC with fewer side effects on patients.

Keywords: *Crithmum maritimum*; hepatocellular carcinoma; sorafenib; drug synergism; plant extracts

Introduction

Hepatocellular carcinoma (HCC) is the sixth leading cause of tumour-related deaths worldwide. Despite the reduced incidence of chronic hepatitis infections, recent projections estimate that it will rise to the third leading cause of Western countries by 2030.[1] The increased incidence of metabolic diseases, such as metabolic syndrome (MetS), diabetes, obesity and non-alcoholic fatty liver disease (NAFLD) explains this concerning epidemiological picture.[2] A direct link between the onset of NAFLD and the development of HCC was suggested.[3] The main approaches to cope with HCC are surgery and, from the pharmacological side, tyrosine-kinase inhibitors, such as sorafenib, regorafenib and levantinib,[2] often in

association with immunotherapeutic drugs, such as pembrolizumab and nivolumab.[4]

Sorafenib, in particular, is an oral multi-kinase inhibitor, which acts on multiple cellular targets, such as cell membrane tyrosine kinase receptors (i.e. PDGFR-b, c-KIT, VEGFR1-3), as well as on downstream intracellular targets, such as members of the RAF/MEK/ERK family pathway (i.e. Raf1).[5, 6] Still, many patients undergoing sorafenib therapy develop acquired resistance, mainly due to sorafenib-induced autophagy.[7] Moreover, the antiangiogenic effect observed after long-term treatment with sorafenib leads to a decreased micro-vessel density resulting in intratumoural hypoxia, which was shown to be correlated to sorafenib resistance.[8] In order to overcome the resistance to sorafenib, a combination of drugs is recommended.[7, 9] Indeed, even such pharmacological protocols present numerous unfavourable consequences on patients and cannot be employed for long-range treatments.[10, 11] Therefore, it is crucial to find new therapeutic opportunities for HCC, which may conjugate efficacy and lower toxicity.

Plant extracts and plant-derived products acquired, in the last years, attention from the scientific community for possible therapeutic applications. Promising effects have been reported in several pathological conditions,[12, 13] including different types of tumours.[14] The interest in this field is still growing, and many research teams are involved in this area of research,[15–17] and the topic was also recently reviewed.[18, 19] We have previously shown that ethyl acetate extract of *Crithmum maritimum* L. (henceforth indicated as CM) harvested in Apulia, displayed a significant anti-proliferative effect in HCC cells.[20] CM is well known back in the past as an edible plant. Recently, CM was re-discovered for its general beneficial health effects,[21] and also for cosmetic applications.[22] CM antioxidant[23] and antimicrobial activities[24] have been described, but we have been the first to prove anti-tumour effects for this plant. In addition, we recently demonstrated that this anti-tumour effect of CM ethyl acetate extract is based on the multi-target effect on tumour cell metabolism, thereby inhibiting the key metabolic pathways involved in tumour cell proliferation.[25]

So, being aware of the potentiality of this plant as a valuable tool for HCC therapy, in this study, we aimed to go further ahead in the investigation of the antitumour potential of CM in HCC by evaluating the possibility that CM ethyl acetate extract administered in combination with half of the IC50 dose of sorafenib result in a similar efficacy to a full dose of sorafenib in HCC cells, and at the same time reduce toxicity. Our hypothesis was to test if a combined treatment with half of the dose of sorafenib that is commonly employed *in vitro*[26, 27] along with CM ethyl acetate extracts results in an outcome similar to that of full-dose sorafenib but with less toxicity. Hereafter, we provide evidence that the combination of sorafenib with the ethyl acetate extract of CM can indeed produce an efficacy comparable to that of full-dose sorafenib, but with reduced toxicity.

In the past few years, research has been conducted on the combined use of sorafenib and plant-derived compounds, such as artesunate,[26] bufalin[27] and capsaicin,[28] and positive results have been obtained. Moreover, an association of four Chinese herbs: *Scutellaria baicalensis* Georgi, *Paeonia Lactiflora* Pall., *Glycyrrhiza uralensis* Fisch. and *Ziziphus Jujuba* Mill, named YIV-906, was found to be effective against HCC in combination with sorafenib by reducing its side effects.[29, 30] Here, we report for the first time in an *in vitro* study that a plant extract from a wild edible plant with no toxic effects can be exploited in combination with sorafenib to reduce its adverse effects, hence giving in perspective future therapeutic opportunities. In addition, we provide insights into the original mechanism of how this combination works. A graphical summary of the results described in the paper is reported in Figure 1D.

Materials and Methods

Plant material and extraction procedure

CM was harvested on the Apulian coast in the metropolitan Bari area (Bari S. Spirito) by experts of the Mediterranean Agronomic Institute (CIHEAM) of Bari. The extraction procedure was performed as previously described.[20] Briefly, after desiccation at 60°C, the plant was pulverized by mechanical trituration. The average yield of exsiccation was ≈94% fresh/dried plant material. The powder was then subjected to a first extraction with hexane (150 ml of hexane for 150 mg plant powder) in a conical flask for 48 h under gentle orbital shaking. The extract was therefore filtered with 'Whatman' filter paper and left to evaporate at room temperature under a chemical hood. Once the powder got desiccated, 150 ml of ethyl acetate was added, and the procedure was reiterated as described above. This extraction protocol was repeated for methanol and ethanol. Obtained extracts were solubilized in cell culture-grade dimethyl sulfoxide (DMSO) (Corning cat. # 25-950-CQC). Extracts were characterized by high-pressure liquid chromatography (HPLC) as previously described.[20]

Chemicals

All of the chemicals and solvents employed in the extraction procedures and analytical determinations are of analytical and HPLC grade. Hexane was purchased from Honeywell. Ethyl acetate, methanol, and ethanol were obtained from Sigma-Aldrich. DMSO is from Corning (cat. # 25-950-CQC).

Cell lines, culturing and treatment

Huh7 and HepG2 cell lines were obtained from JCRB cell bank (cat. # JCRB0403 and cat. # JCRB1054, respectively). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1 g/L glucose, 4 mM glutamine, 1 mM sodium pyruvate (Corning cat. #10-014-CVR), supplemented with 1× MEM-Nonessential Amino Acids (Corning cat. # 25-025-CIR), 20 mM Hepes Buffer (Aurogene cat. # AU-L0180-500), 1× Antibiotic-Antimycotic solution (Corning cat. # 30-004-CI) and 10% foetal bovine serum (FBS) (Corning cat. # 35-079-CV). Cells were cultivated under standard culturing conditions (humidified atmosphere, 37°C and 5% CO_2).

Cells were treated 24 h after plating for all of the experiments described. Sorafenib and CM ethyl acetate extract were added together in the cell culture medium. For cell growth assays cells were treated for 72 h, while for the determination of cell growth kinetics, we treated cells 24 h after plating and we counted cells every 24 h for 120 h.

For cell cycle and apoptosis analyses, cells were treated for 48 h, while for immunoblotting analyses of signalling proteins, cells were treated for 30 min.

Cell growth assays

End-point proliferation was determined by crystal violet staining 72 h after treatment. Crystal violet (Sigma-Aldrich cat. #C3886) was diluted in EtOH/H_2O 10% v/v to obtain a 1 mg/ml solution. After fixation in 4% paraformaldehyde, crystal violet was added to cells. The colour was eluted using 10% acetic acid and absorbance was

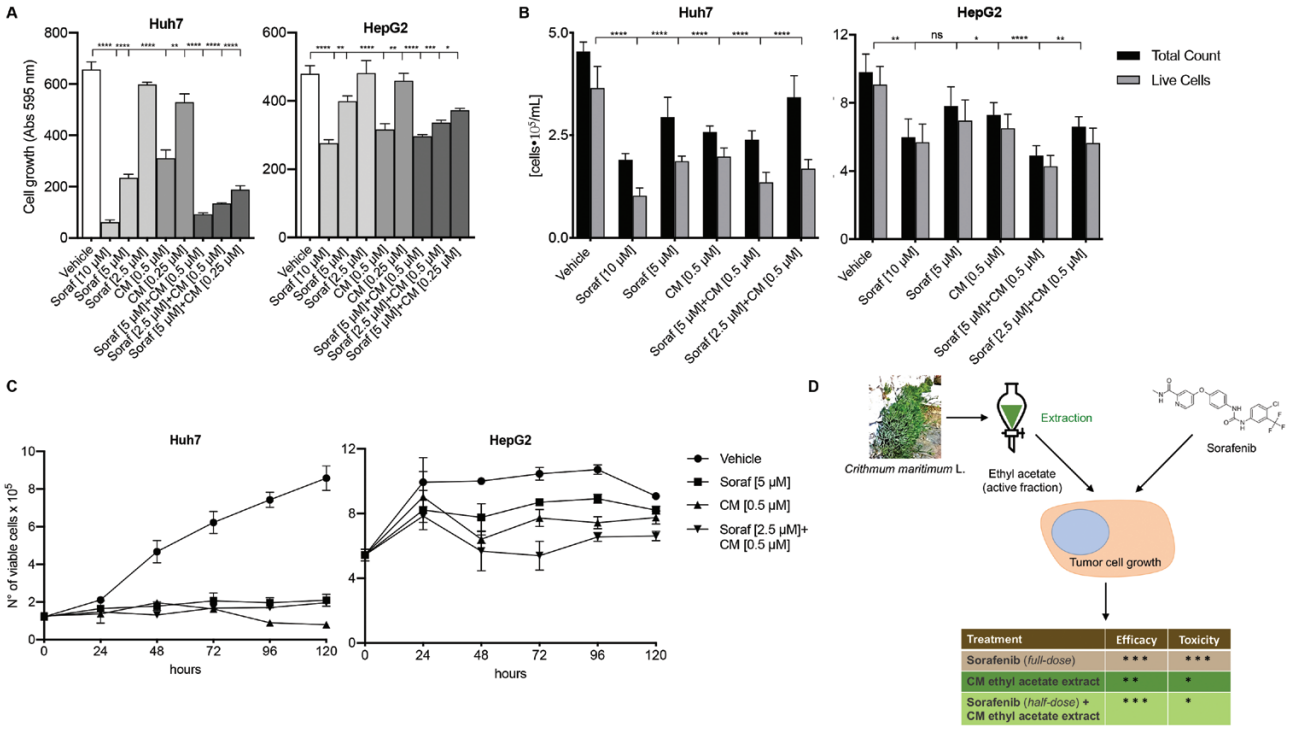

Figure 1 Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on HCC cell growth. (A) The effect of the combined treatment on HCC cell growth was assessed by crystal violet staining after a 72-h treatment. (B) The effect of the combined treatment on HCC cell growth was assessed by trypan blue exclusion staining after a 72-h treatment. (C) Growth kinetic curve showing the effect of the combined treatment on HCC cell growth. Data represent mean ± SEM of three independent experiments performed in duplicate. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$. (D) Graphical summary of the findings of this study illustrating the advantages of the combined treatment with CM ethyl acetate extract and half-dose sorafenib for the inhibition of HCC cell growth.

measured at λ = 595 nm using an iMark plate reader (Bio-Rad cat. #168–1135).

End-point counting was performed by Trypan blue exclusion test 72 h after treatment.

Determination of cell growth kinetics

The determination of cell growth kinetics was performed by treating cells when ~30–35% confluent and by counting the number of cells every 24 h for 120 h. Cell counting was done using the Trypan blue exclusion test.

Cell cycle analysis

Cell cycle analysis was performed employing a Guava EasyCyte benchtop flow cytometer (Merck cat. #0500-5009) using the Guava Cell Cycle Assay (Merck cat. #4500-0220) following producers' directions of use. Additional details are reported in the Supplementary Information.

Immunoblotting analyses

Cell lysates were prepared using the Cell Signaling lysis buffer (Cell Signaling cat. #9803) supplemented with protease and phosphatase inhibitors (Roche cat. #04693159001 and 04906837001). Samples were prepared with Laemmli Sample Buffer (BioRad cat. #161–0737) supplemented by beta-Mercaptoethanol. Proteins were separated by SDS-PAGE and then blotted onto nitrocellulose membranes. Primary antibody incubation was performed overnight at 4°C with gentle shaking using the antibodies reported in the Supplementary Information. Membranes were acquired using Licor c-Digit. Each panel of the blots reported in Figure 4A–D was obtained by stripping the same membrane two times and re-probing it with the different antibodies indicated.

Apoptosis analysis

Apoptosis was evaluated using a Guava EasyCyte benchtop flow cytometer (Merck cat. #0500-5009) using the Guava "Nexin" kit (Merck cat. #4500-0450) following producers' directions of use. Additional details are reported in the Supplementary Information.

Statistical analyses

The normality of data was verified with D'Agostino-Pearson's Omnibus K2 test. One-way analysis of variance followed by Dunnett's *post-hoc* test was used to determine statistical significance when data were normally distributed. Otherwise, the Kruskal–Wallis test followed by Dunn's multiple comparisons test was used. Statistical analyses and graphs were performed with GraphPad Prism 9 software.

Results

The combination of CM ethyl acetate extract and half-dose sorafenib decreases the growth of HCC cells, similar to full-dose sorafenib

To evaluate the effect of the combined treatment with CM ethyl acetate extract and sorafenib, we first assessed cell proliferation in two HCC cell lines: Huh7 and HepG2. To this end, we performed a crystal violet staining assay and evaluated the number of viable cells by the Trypan Blue dye exclusion test after 72 h treatment. Also, we built growth kinetic curves in the same cell lines in an interval of

time of 120 h treatment, treating cells 24 h after plating. Preliminary, for crystal violet staining assay, we treated cells with three different concentrations of sorafenib, 10, 5 and 2.5 μM, and with two concentrations of CM ethyl acetate extract, 0.5 μM and 0.25 μM. Also, we combined 5 μM sorafenib with 0.5 μM and 0.25 μM CM ethyl acetate extract (Figure 1A). Considering the results obtained, in the following experiments we focused only on CM 0.5 μM. Results reported in Figure 1A and B show that CM ethyl acetate extract effectively cooperates with sorafenib in inhibiting HCC cell growth. The combination of 5 μM sorafenib with 0.5 μM CM ethyl acetate extract reduced HCC cell proliferation similarly to 10 μM sorafenib. Likewise, in both cell lines, 5 μM sorafenib combined with 0.25 μM CM ethyl acetate extract was as effective as 5 μM sorafenib. Therefore, we analysed the effect of the combined treatment on growth kinetics. Here, we treated both cell lines with sorafenib 5 μM, CM ethyl acetate extract 0.5 μM and with a combination sorafenib 2.5 μM plus CM 0.5 μM, and we followed cell growth for 5 days. In both cell lines, the combined treatment shows an efficacy comparable to that shown by individual treatments (Figure 1C). Proliferation experiments were corroborated by viability assays. Data obtained by sulforhodamine B (SRB), MTT and neutral red assay in Huh7 and HepG2 are shown in Supplementary Figure S1.

CM ethyl acetate extract combined with half-dose sorafenib inhibits cell cycle progression similar to full-dose sorafenib without increasing toxicity

Next, we focused on the effect of the combination of CM ethyl acetate extract and sorafenib on the progression of the entire cell cycle. To this purpose, after 24 h serum starvation, we treated Huh7 and HepG2 cells for 48 h with 10 μM and 5 μM sorafenib, 0.5 μM CM ethyl acetate extract, and with the combination of 5 μM sorafenib plus 0.5 μM CM ethyl acetate extract. Results reported in Figure 2 present clear evidence that, in both cell lines, the combined treatment results in an effect similar to that produced by the full-dose sorafenib and by 0.5 μM CM ethyl acetate extract, with an increase of the percentage of cells blocked in the G1 phase. These data provide a mechanistic basis for the observed effects on cell growth reported in Figure 1. Next, we analysed the effect of the combination on cytotoxicity employing the Guava 'ViaCount' reagent, which allows determining cell number, viability and apoptosis exploiting the different permeability properties of two DNA-binding dyes. Cells were treated for 72 h with sorafenib, CM and their combination at the indicated concentrations. Results reported in Supplementary Figure S2 show that similar to the full dose of sorafenib, the combination reduces the number of cells and it is less toxic than the full dose of sorafenib.

CM ethyl acetate extract in combination with half-dose sorafenib stimulates apoptosis but not necrosis like full-dose sorafenib

Then, we analysed the effect of the combined treatment on apoptosis by evaluating phosphatidylserine externalization employing a cytofluorimetric approach. In this way, we could distinguish early apoptotic cells (Annexin V+/7AAD-), late apoptotic cells (Annexin V+/7AAD+), and cell debris (Annexin V-/7AAD-). Results reported in Figure 3 show that, in both cell lines, the combined treatment reduced cell number similarly to full dose sorafenib (Figure 3C and F), but with much fewer toxic effects (Figure 3A–B and D–E). Moreover, in both cell lines, the combined treatment induced an early apoptotic state. This effect is especially evident in Huh7 cells, whereas in HepG2 is less pronounced. Of note, in both cell lines, the distribution of cells after the cytofluorimetric sorting looks different in cells treated with CM ethyl acetate extract and with the combination compared to the cells treated with sorafenib (Figure 3B and E). CM may induce peculiar effects on the regulation of the apoptotic process worth to be taken into consideration in future experiments. To corroborate these observations, we performed nuclear DAPI staining in Huh7 cells. The data reported in Supplementary Figure S3A–E confirm the data reported in Figure 3A, i.e. there are fewer cells in late apoptosis/necrosis in cells treated with the combination compared

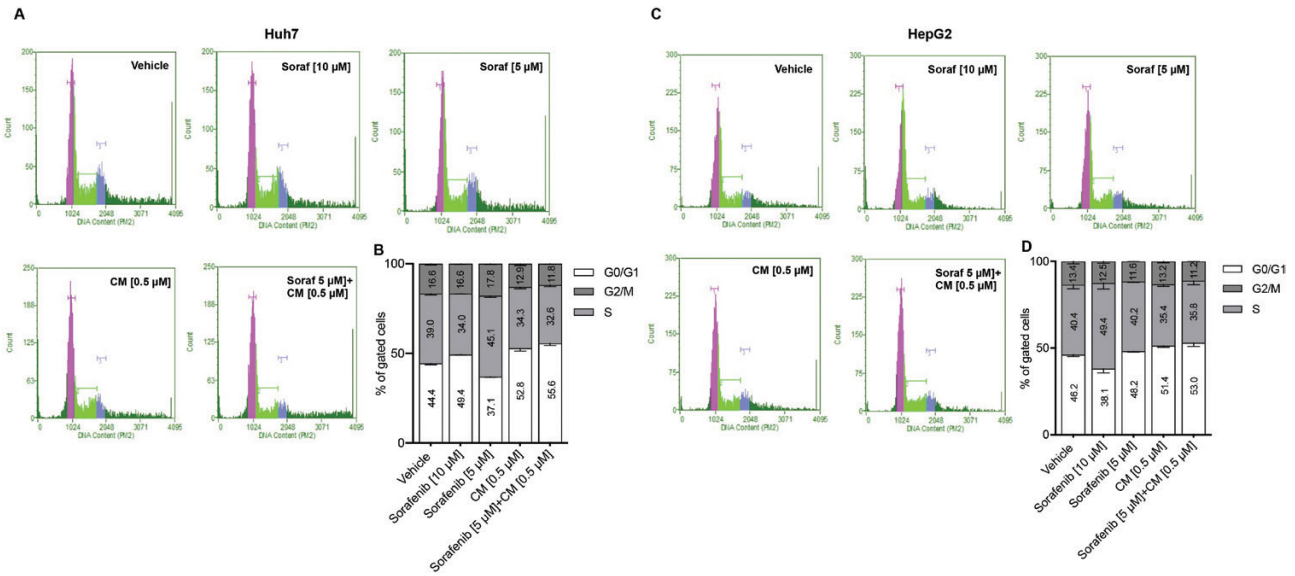

Figure 2 Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on HCC cell cycle regulation. (A) Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on HCC cell cycle regulation in Huh7 cells. Cell cycle histograms are representative of one experiment. (B) Data represent two biological replicates performed in duplicate. (C) Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on HCC cell cycle regulation in HepG2 cells. (D) Data represent two biological replicates performed in duplicate.

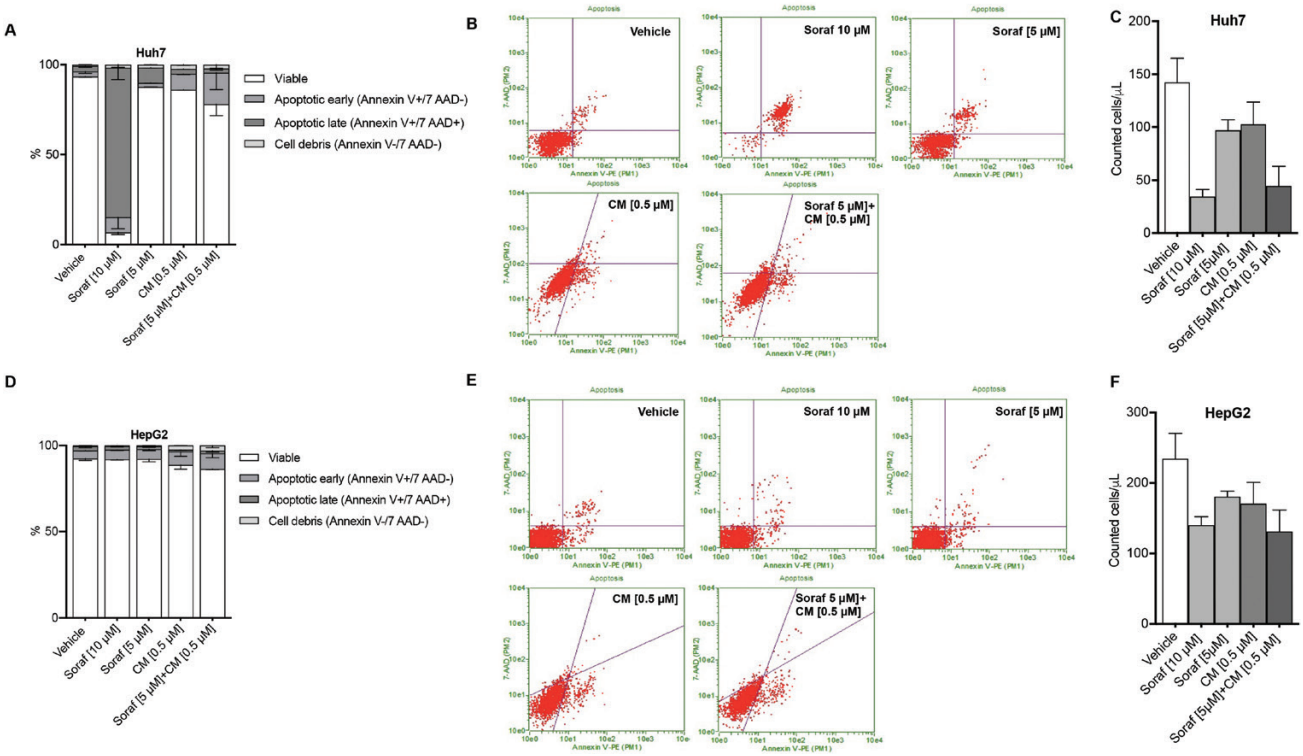

Figure 3 Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on HCC cell apoptosis. (A) Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on HCC cell apoptosis in Huh7 cells. Data represent two biological replicates performed in duplicate. (B) Results are representative of one experiment. (C) Data report the number of cells/µL relative to the experiments in (A). (D) Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on HCC cell apoptosis in HepG2 cells. Data represent two biological replicates performed in duplicate. (E) Results are representative of one experiment. (F) Data report the number of cells/µL relative to the experiments in (D).

with those treated with the full dose of sorafenib. It is possible to distinguish cell nuclei in the early stages of apoptosis, which corresponds to what has been shown by cytofluorimetric experiments.

CM ethyl acetate extract in combination with half-dose sorafenib regulates the phosphorylation of key cell cycle- and apoptosis-regulating proteins similarly to full-dose sorafenib

To further substantiate the observations reported above and to get further insights into the mechanisms by which the combination of CM ethyl acetate extract with sorafenib exerts its effects, we considered four key proteins whose phosphorylation is involved in the control of cell proliferation and apoptosis, namely p44/p42 MAPK (ERK 1/2), p38 MAPK, Akt and Bad. Since it has been shown that HepG2 cells have low levels of basal ERK 1/2 phosphorylation and that Akt is not responsive to sorafenib,[31] we decided to show only results obtained in Huh7 cells. We anyway performed the analysis in HepG2 cells as well (Supplementary Figure S4). In particular, it has been shown that in Huh7 cells ERK 1/2 and p38 MAPK phosphorylation is decreased, whereas Akt phosphorylation is increased upon treatment with sorafenib.[32] Likewise, Bad phosphorylation is downregulated by sorafenib in Huh7 cells, while the level of Bad is upregulated.[32] Here, we found in Huh7 cells that the combination of CM with 5 µM sorafenib exerts an effect similar to that of full-dose sorafenib, thus corroborating our observations on cell cycle regulation and apoptosis. Interestingly, we found that treatment with CM alone determines a stronger Akt phosphorylation compared to 10 µM sorafenib. These findings provide interesting insights and possibilities for further research in the future.

Discussion

The use of plants in disease prevention and treatment has attracted more and more attention from the scientific community.[33] For example, plant-derived molecules are being evaluated as potential anti-cancer drugs,[34] and some are being tested in clinical trials.[35] Overall, there is growing interest by the scientific community.[18, 19] Several shreds of evidence also support the view that therapeutic health effects are mainly due to the synergistic effect between the complex assortment of compounds distinctive of each plant, rather than to the action of a single or few molecules.[36, 37] Anticancer effects of extracts of whole desert plants have been described in HCC cells.[38] Similarly, it is reported that the ethyl acetate extracts of three traditional plants are effective against cervical and liver cancer.[39, 40]

We have previously described that CM ethyl acetate extract has a significant cytostatic effect in HCC cell lines,[20] and this effect is attributed to its multi-target action on HCC cell metabolism.[25] In this study, we aimed at further characterizing the possibility of using CM for therapeutic applications in HCC. In particular, we explored the effect of CM in combination with sorafenib, intending to decrease the dose of sorafenib to reduce its adverse effects. Our data indicate that the combination of half of the IC50 sorafenib dose usually employed *in vitro* experiments (10 µM)[41] with 0.5 µM CM

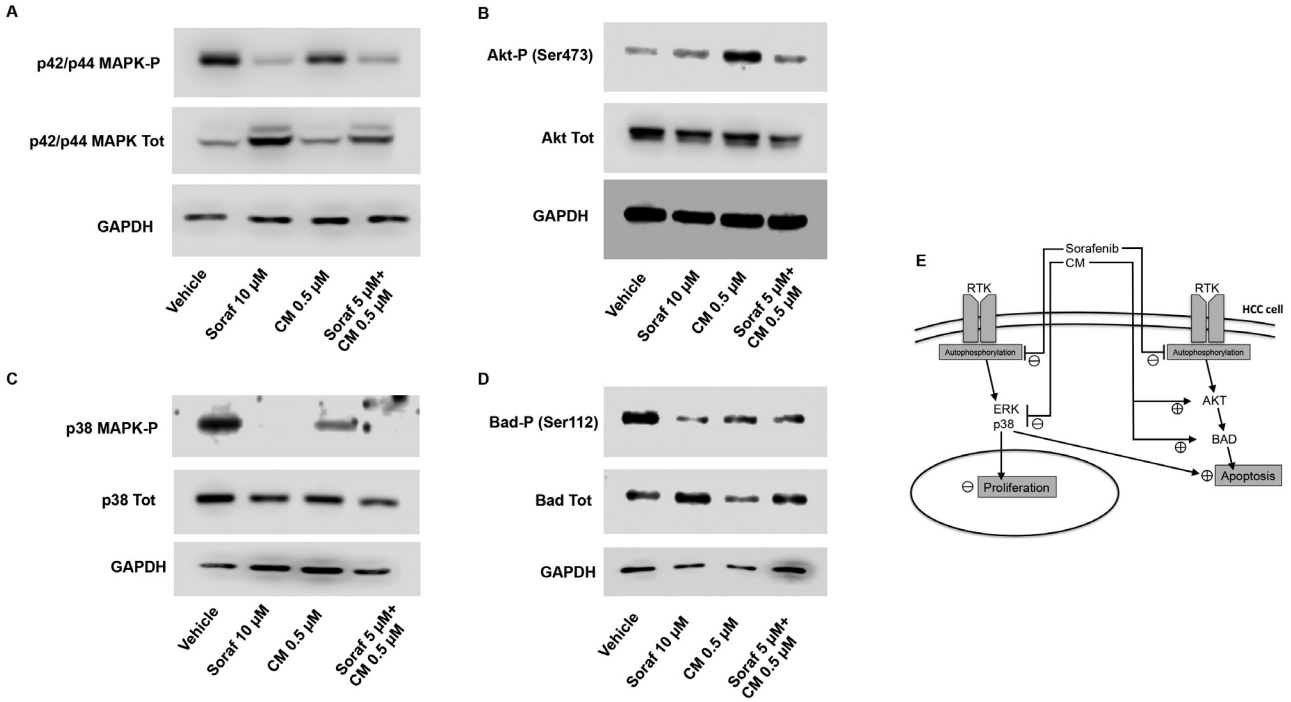

Figure 4 Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on the phosphorylation and expression of key proteins involved in the regulation of cell cycle and apoptosis in Huh7 cells. (A) Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on p42/p44 MAPK phosphorylation and expression. (B) Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on Akt phosphorylation and expression. (C) Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on p38 MAPK phosphorylation and expression. (D) Effect of the combined treatment with CM ethyl acetate extract and half-dose sorafenib on Bad phosphorylation and expression. For each blot, two biological replicates and two technical replicates were performed. (E) Graphical summary of the interaction between CM ethyl acetate extract and sorafenib in signal transduction pathways that control cell proliferation and apoptosis. RTK = Receptor tyrosine kinase; ⊕ = Stimulation; ∅ = Inhibition.

ethyl acetate extract is as effective as full-dose sorafenib in inhibiting HCC cell growth, but with fewer toxic effects. This observation was substantiated by data on the regulation of cell cycle and apoptosis, where an increase in the percentage of cells blocked in the G1 phase was observed together with the promotion of apoptosis and a reduction of necrosis. We believe that the last point is particularly interesting because the combination therapy has the added value of significantly reducing the toxic effect, so it has the advantage of inhibiting cell growth and greatly reducing the effect on cell death. We can speculate that some components of CM ethyl acetate extract can exert a proapoptotic effect in HCC cells or can synergize with sorafenib in a still unknown way. Future researches will be planned to clarify this important point.

As mentioned in the Introduction, several works recently reported the effectiveness of the combination of sorafenib with plant-derived compounds, such as capsaicin,[28] bufalin[27] or artesunate.[26] Similarly, the combination of sorafenib with YIV-906 (a mixture of four Chinese herbs) was found to be effective for HCC, even if some side effects were described.[29, 30] Even so, we are the first to report on the efficacy of sorafenib in combination with an edible plant extract.

From a general point of view, tumours use several mechanisms to develop resistance to drug treatments. A generally recognized mechanism that plays an important role in drug resistance is the acidic pH of the tumour microenvironment.[42] Interestingly, we have previously shown that the ethyl acetate extract of CM is still effective under such acidic conditions.[25] From a future point of view, it will be interesting to test the combined treatment effect of CM plus sorafenib under this acidic condition. As mentioned in the introduction,[5, 8] this will confirm whether CM ethyl acetate extract can overcome acid-induced resistance to sorafenib. Indeed, it has been shown that sorafenib-induced hypoxia leads to the development of acidic pH conditions in the microenvironment.[43] Another important mechanism of acquired tumour drug resistance is through the connection of ERM (Ezrin/Radixin/Moesin) proteins to the cell membrane, which determines the multidrug resistance (MDR) phenotype at the cellular level. This mechanism was shown to be mediated by P-glycoprotein interaction with the actin cytoskeleton in two tumour cell lines.[44, 45] These mechanisms are stimulated in acidic microenvironment pH conditions, thus suggesting that a combination of proton exchanger inhibitors could prove useful.[46] In our perspective, this gives us the rationale to further investigate in the future the effect of the combination of sorafenib plus CM in acidic pH conditions. This may provide important information about the possible role of CM ethyl acetate extract in counteracting MDR.

Conclusions

Overall, our data provide solid preclinical evidence that combined treatment with a reduced dose of the traditional chemotherapy drug sorafenib and an extract from the wild and edible plant CM can effectively block the growth of HCC cells while reducing sorafenib toxic effects. Figures 1D and 4E outline the key points described in this article. Such a combined approach provides a scientific basis for the development of new effective options for the treatment of liver cancer with reduced drug toxicity. In conclusion, our findings pave the way for a comprehensive treatment of HCC with fewer side effects. Future research will allow us to further develop this opportunity. In particular, we will corroborate the findings of the present

study by expanding the repertoire of the *in vitro* models used, hence using more HCC cell lines. As an additional opportunity, we will explore the effect of CM alone and in combination with sorafenib in other tumour cell lines as well as *in vivo* models. In this perspective, it will be also interesting to test whether the combined approach could prove effective in targeting the metastatic process, which is one of the main challenges in the treatment of tumours and for which new pharmacological approaches are extremely needed.[47] Clinical trials on patients may be also be planned. Finally, it is worth to be mentioned here that our combined approach may benefit from innovative methods of delivery, such as nanosized drug delivery systems, for example, targeted nanoparticles that would bind HCC-specific targets.[48]

Supplementary Material

Supplementary data are available at *Journal of Pharmacy and Pharmacology* online.

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Author Contributions

D.G. performed the experiments, elaborated data and wrote the manuscript; F.C. performed the experiments; G.C. provided reagents; G.F. revised the manuscript; C.S. revised the manuscript; A.M. conceived the study and supervised the experiments, wrote and revised the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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