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Elettaria Cardamom Extract Synergistically Enhaning Sorafenib-Induced Apoptosis in HepG2 Cells

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ABSTRACT

Introduction: *Liver cancer is ranked as the second most common cause of death globally as a result of its poor prognosis. It can be treated with sorafenib, but its use is limited due to its toxicity and adverse reactions. Lower doses of sorafenib with other complementary agents are recommended to minimize toxicity. Cardamom seeds are one of the most common ingredients of Indian and Chinese traditional medicine, and different studies have suggested that cardamom extract can display anti-cancer activities.* ***Aim:*** *this study aims to investigate the efficiency of Elettaria Cardamom Extract (ECE) on enhancement of Sorafenib-induced apoptosis in HepG2.* ***Methods:*** *Human liver cancer cell line (HepG2) were exposed to increasing concentrations of individual and combined treatments of Sorafenib and ECE for 24 h. The viability of cells was examined using MTT Assay. Clonogenicity and cell migration assays were carried out. Reactive oxygen species (ROS) generation and mitochondrial membrane potential (MMP) level were determined by DCFH-DA and JC-1 dye, respectively. Agarose gel electrophoresis and comet examinations were carried out to estimate the DNA damage.* ***Results:*** *Combined treatment of ECE with sorafenib suppressed the proliferation, colony formation and cell migration of HepG2 cells more than the sorafenib did alone. The half maximal inhibitory concentration (IC50), after 24h of incubation were 15 µM of sorafenib and 9 and 7.3 µM of sorafenib enhanced by 5 and 10 µg / 100 µl of ECE respectively. HepG2 treated cells displayed biochemical features of apoptotic cell death. The combined treatment increased the ROS production, reduced the level of MMP, increased Comet tail length and induced DNA fragmentation more than sorafenib did alone.* ***Conclusions:*** *These findings demonstrate that ECE enhanced the sorafenib effect in HepG2 cells and suggest that the ECE may be a promising agent for reducing sorafenib side effects in hepatocellular carcinoma (HCC).*

Key words: *Hepatocellular Carcinoma, Monoterpenes, Apoptosis, Sorafenib.*

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most primary cancer of the liver and occurs in about 70 % to 90 % of patients suffering from liver cancer [1]. HCC is linked to some of chronic liver diseases such as infections with hepatitis B (HBV) or hepatitis C (HCV) [2, 3]. Obesity and diabetes mellitus have also been correlated with

increased risk of HCC [4]. Alcohol is another important risk factor for developing HCC with increased risk as levels of alcohol intake rise [4, 5]. In some regions of the world, especially in Asia and Africa, aflatoxin is the major risk factor of HCC [6]. Liver cancer prognosis is very poor, making it the second most common cause of death. The estimated death count in 2012 was approximately 746,000 (9.1 % of the total cancer death) [7]. Sorafenib is a small-sized molecule which inhibits tumor-cell proliferation and tumor angiogenesis and can lead to increase apoptosis in a huge number of tumor models, such as the HCC [8]. Some limitations of sorafenib use are its toxicity and adverse effects [9-11]. To reduce sorafenib-induced toxicity, it has been recommended to use lower doses of sorafenib with other complementary agents [12]. Nowadays, some folk and traditional medicines use a range of natural substances and plants including herbs, spices and vegetables as new drugs to treat cancer [13, 14].

Among popular spices, Elettaria cardamomum L. (Zingiberaceae) or cardamom can be named. It is known as the "queen of spices", and is frequently used in Easter, Arab, Scandinavian as well as western cuisines [15]. The extract of E. cardamom has been reported to generate therapeutic effects. The main constituents of cardamom are monoterpenes: 1,8-cineole, α-terpineol, citronellol, α-phellandrene, sabinene, myrcene, borneol, camphor, γ-terpinene, p-cymene, terpinolene, linalool, and α and β pinene [16]. Several experimental studies revealed the anticancer properties of cardamom [17-20]. Monoterpenes are non-toxic and have been recommended to treat several diseases including diabetes [21], cancer [22], and hypercholesterolemia [23, 24]. Certain monoterpenes exhibit antiproliferative, and proapoptotic activities in cell culture [22, 25]. Accordingly, herbal extracts are pharmaceutically more potent compared to the purified components [26, 27]. The present study was conducted to explore the potential of ECE to potentiate the action of Sorafenib using human liver cancer cell line, HepG2, and further to elucidate the apoptosis features of this combination.

MATERIALS AND METHODS

Agents

Sorafenib, ECE (the product has vegetable palm glycerin (VPG) in content) and VPG were purchased from Hangzhou Yuhao Chemical Technology (Co., Ltd. CAT No. RT4715), Hawaii Pharm LLC [28] and [29], respectively. For the in vitro assays, ECE and VPG were ready to be used; while, Sorafenib was dissolved in 100 % DMSO and diluted in DMEM to the required concentrations with a final DMSO concentration of 0.1 % [30].

Cell culture

The Human HCC (HepG2) cell line was provided from King Fahad Center for Medical Research, King Abdulaziz University, Jeddah, Saudi Arabia. HepG2 cells were grown using Dulbecco's modified Eagle's medium (DMEM, Gibco.) with 10% fetal bovine serum (FBS, Sigma.) and 1% penicillin-streptomycin antibiotics (Gibco) and incubated in tissue culture flask under a humidifying atmosphere with 5% CO_2 and 95% air at 37°.

Cell viability, clonogenic and cell migration assays

To investigate the effects of single (either sorafenib, VPG or ECE) and combined (ECE plus sorafenib) cells were treated with (3, 5, 7, 10, 15, 20 and 30 µM of sorafenib), (1, 5, 7 and 10 µl/100 µl of VPG) and (1, 5, 10, 20, 30 and 50 µg/100 µl of ECE) and combined (5 and 10 µg/100 µl of ECE with 3, 5, 7 and 10 µM of Sorafenib). Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5- Diphenyltetrazoliumbromide (MTT) [31]. Briefly, HepG2 cells were seeded onto 96 well plates (7.5×10^3 cells/well) and grown overnight. The cells were then treated with concentrations of single (Sorafenib, VPG and ECE) and combined (5 and 10 µg/100 µl of ECE with Sorafenib) treatments and incubated for 24 h. Then MTT assay was performed by adding 10 µl of MTT (5 mg/ml, Goldbio, Catalog.T-030-1) to the cells for 3-4 h. The culture medium which contained the MTT was discarded. The dye crystals were left to dissolve in the Dimethyl sulfoxide (DMSO). A wavelength of 570 nm was used to measure the absorbance of the converted dye. The high absorbance is directly proportional to the viability of cells.

The clonogenic assay was used to measure tumor cells' ability to grow and form foci unrestrictedly by the growth contact inhibition as is the case with the normal cells which experience no transformation [32]. Around $2.5x10^3$ cells were seeded into six-well plates in triplicate and allowed to adhere during the night. Then, cell culture medium was aspirated off, and cells were treated with ECE and sorafenib in increasing single and combined concentrations. The cells were then incubated at temperature 37 °C undisturbed for ten days to allow surviving cells to proliferate and form colonies. On the tenth day, the colonies were washed with cold Dulbecco's phosphate-buffered saline (DPBS), fixed with ten percent formalin-based buffer and 0.25 % Giemsa stain.

For Cell Migration Assay, cells were seeded in 24 well plate 35×10^3 cells/well and incubated for 24 h. A pipette was used to produce a wound in the cell's monolayer, media were aspirated off, and cells were treated using single (25 µg/ml of ECE and 1, 3, 5 and 7 µM of Sorafenib) and combined treatments. The wound healing (cell migration) was recorded by taking pictures at two time points: 0 and 24 h.

Determination of ROS Production

To assess the ROS generation, 2′,7′-Dichlorodihydrofluorescein diacetate (DCFHDA) (Cayman Chemicals, USA) was used. HepG2 cells were treated with single\combined treatment for twenty-four hours. The cells were then labeled with DCFH-DA for half an hour and washed with PBS. A micro-plate reader (BioTek Synergy) was then used to measure fluorescence intensity (ex:485/20 nm and em: 528/20 nm).

Analysis of Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP) was assessed using JC-1 dye (Cayman Chemicals, USA). In brief, the cells of HepG2 were cultured in 96-well plate (7.5×10^3 cells/well), treated with concentrations of single (ECE and Sorafenib) and combined (5 µg/100 µl of ECE with Sorafenib) treatments for twenty-four hours. Then, cells were labeled with JC-1 dye for half an hour and then washed in PBS. A micro-plate reader (BioTek Synergy) was used to measure the fluorescence intensity (ex: 485/20 nm and em: 528/20 nm).

Single-cell gel electrophoresis (Comet assay)

Comet assay (Single-Cell Gel Electrophoresis) was carried out to identify the DNA damage. Concentrations of single (ECE and Sorafenib) and combined (0.1 mg/ml of ECE with Sorafenib) were added to the cells for twenty-four hours in complete medium, harvested, re-suspended in ice-cold PBS and placed under dim light as recommended by Elkady, Hussein [33].

DNA fragmentation assay

DNA gel electrophoresis was utilized to assess the internucleosomal DNA cleavage, as described by Elkady, Hussein [34].

Statistical analyses

All experiments were conducted in triplicates. Megastat 12 was used to perform statistical analysis. The findings are presented as mean ± SD. To Identify the differences among the samples, one-way analysis of variance (ANOVA) was used. The statistical significance was measured at the level of $P \leq 0.05$.

RESULTS

Sorafenib and ECE inhibiting HepG2 cell growth

Treatment of HepG2 cells by combined treatments significantly ($P \leq 0.05$) decreased cell viability compared to individual treatments. The effect on cell viability was concentration-dependent (Figure 1. A and D). ECE decreased HepG2 cell viability (Figure (1.C); whereas, VPG (Figure 1.B) had no effect on HepG2 cell viability, which means its effect was excluded from ECE action and did not interfere with the ECE-induced effects. Compound potency was measured using half-maximal inhibitory concentration (IC_{50}). In this study, sorafenib IC_{50} was decreased by treatment in combined with the ECE dose. According to the results obtained from the MTT assay, the IC50 values were 15, 9 and 7.5 µM of sorafenib only, with 5 µg and 10 µg/100 µl of ECE, respectively (Figures 1. A and D), and the IC_{50} value of ECE was 26 µg/100 µl (Figure 1.C).

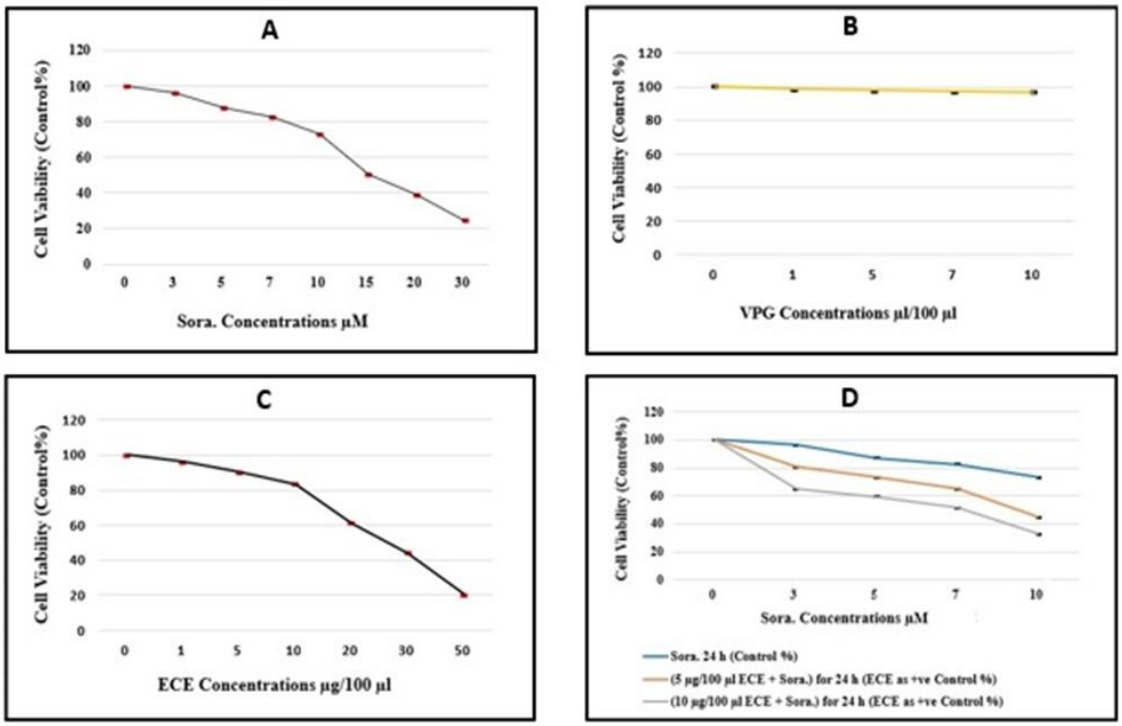
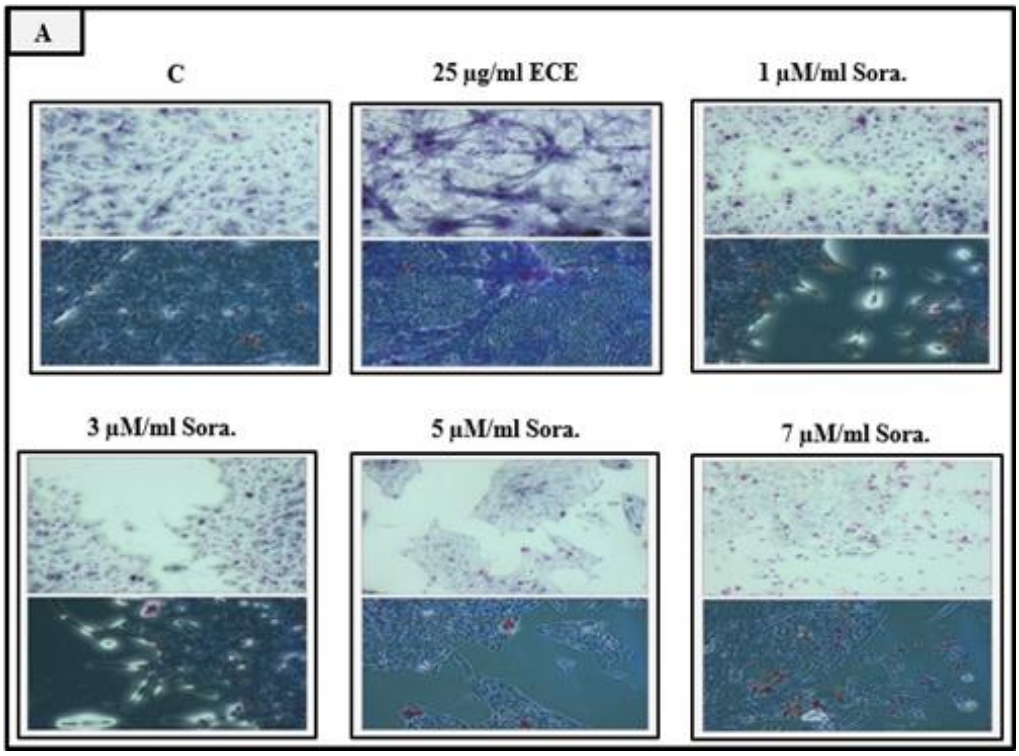


Figure 1. Effect of A) Sorafenib, B) VPG, C) ECE and D) 5 and 10 µg/100 µl ECE with sorafenib on cell viability, HepG2 cells (7.5×10^3 cells/well) following 24 h of treatment

Combined treatment decreasing colonies formation

The antiproliferative potential of the single and combined treatments on HepG2 cells was determined and verified by the colony formation assay. The combined treatment (Figure 2.B) gradually decreased the amount and size of rising colonies in a dose-dependent manner compared to single treatment (Figure 2.A). Control colonies grew forming a continuous sheet monolayer that covered the whole surface; while, in treated cells, the cells appeared to be less in number and there were considerable spaces between them. In addition, the cells treated with the combined concentrations (25 µg/ml ECE with 7 µM/ml sorafenib) were rounded and dark, which indicates cell death (Figure 2.B).



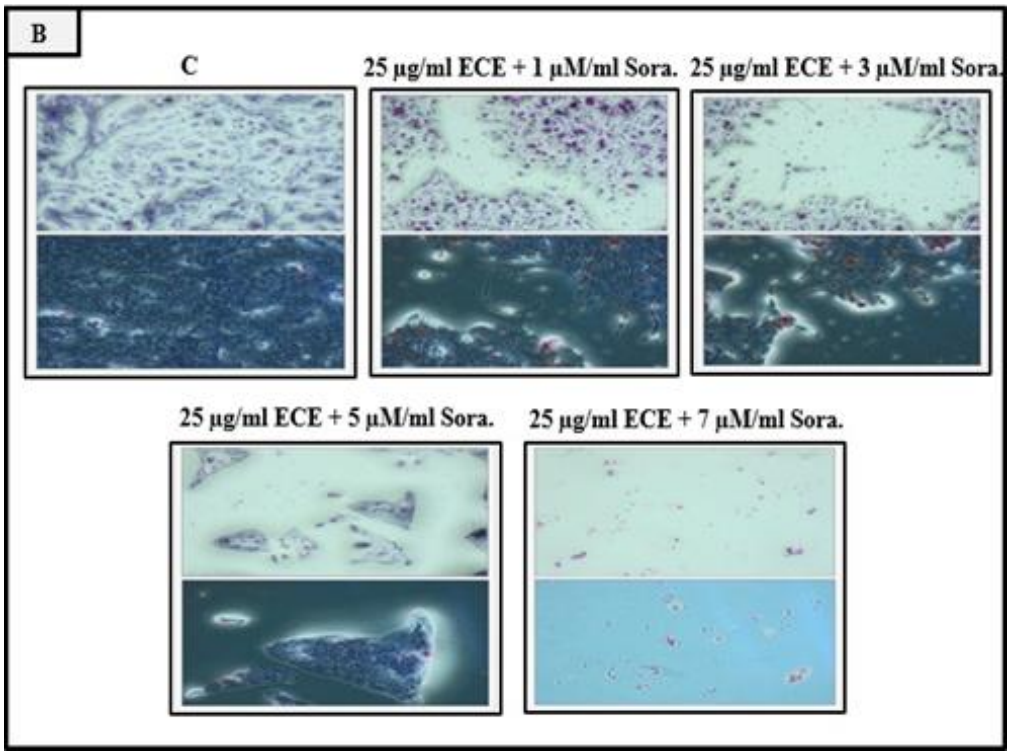


Figure 2. Clonogenicity assay results of A) single and B) combined treatments on the growth of HepG2 cells (2.5×10^3 cells/well) following 10 days of treatment. Cells were observed under light microscopy magnification x4 and x10.

Inhibition of cell migration by combined treatments

A wound healing assay was performed to study directional cell migration. This method allows us to observe cells and how they are reacting. A wound was generated using a pipette tip in a cell monolayer. The wound healing (cell migration) was recorded by taking pictures at two-time points: 0 and 24 h. After 24 h, the control showed that the cells had started to migrate and the wound disappeared (Figure 3); while, in the treated cells the wound remained large and was more apparent in combined treatment cells (Figure 3.B) than the single treatment cells (Figure 3.A). In addition, in the 24 h single treatment cells (1, 3, 5 µM of sorafenib and 25 µg/ml of ECE) have formed a semi-continuous sheet monolayer; whilst, at the sorafenib concentration of (7 µM) the wound remained large and apparent. In the combined treatment, for all doses the wound remained large and apparent.

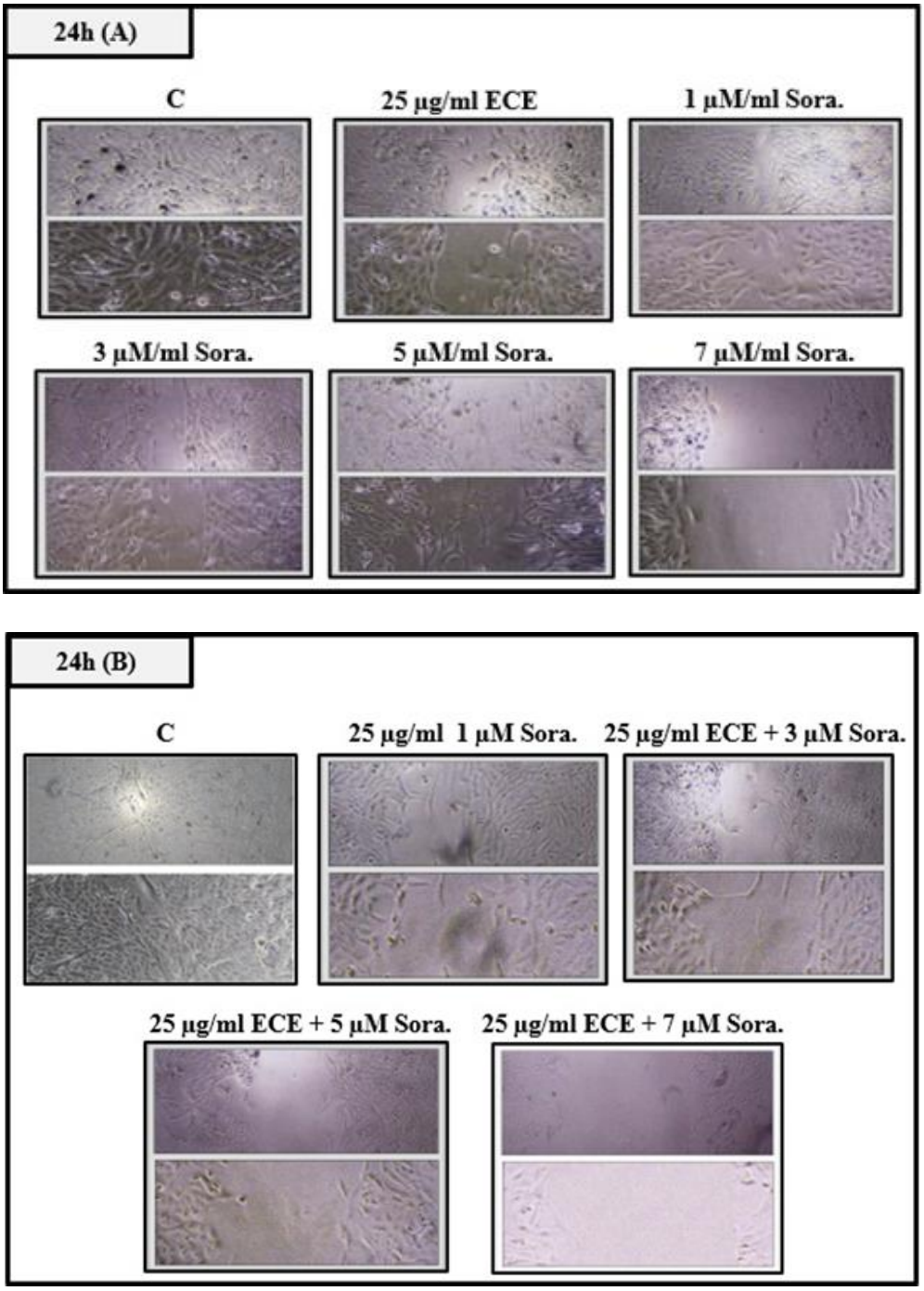


Figure 3. The cell migration assay for A) single and B) combined treatments on HepG2 cell, showing combined treatment (25 µg/ml of ECE with 1, 3, 7 and 10 µM of sorafenib) inhibited cell migration more than the single treatment in HepG2 cells after 24 h. Cells were observed under light microscopy magnification x4 and x10.

Increased generation of ROS

To study whether single and combined treatment induce apoptosis through the production of ROS, the intracellular level of ROS was measured by using a fluorescent dye DCFH-DA. The findings (Figure 4) showed that the combined treatment induces a dose-dependent ROS production ($P < 0.05$) more than the single treatment. In addition, ROS accumulation encourages outer mitochondrial membrane permeabilization, driving to damage of MMP in the combined treatment more than the single treatment (Figure 5). This suggests that ECE enhanced the sorafenib action.

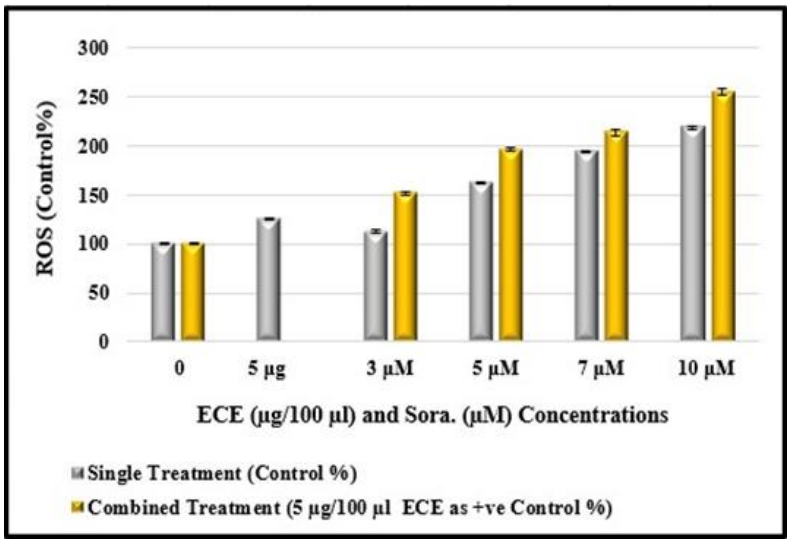


Figure 4. Single and combined treatments induce reactive oxygen species (ROS) production in HepG2 cells in a dose-dependent manner. P<0.05 is significantly different compared with the control.

Single and combined treatments induce DNA damage

Among all the markers of apoptosis, DNA fragmentation is considered a hallmark. Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. This ocuurs due to different apoptotic stimuli in a wide range of cell types [35]. To examine whether single and combined treatments cause fragmentation, a Comet assay was performed (Figure 6). The nuclei and DNA in the untreated cells showed round with no tail; whilst, concentration of ECE (0.1 mg/ml) and sorafenib (20 µM) showed the point in time of DNA migration out of the nucleus that resulted from DNA breakage and the comet tail of treated-cells appeared. Combined treatment (0.1 mg/ml of ECE with 20 µM of sorafenib) showed the occurrence of a comet with a shining head and a longer tail.

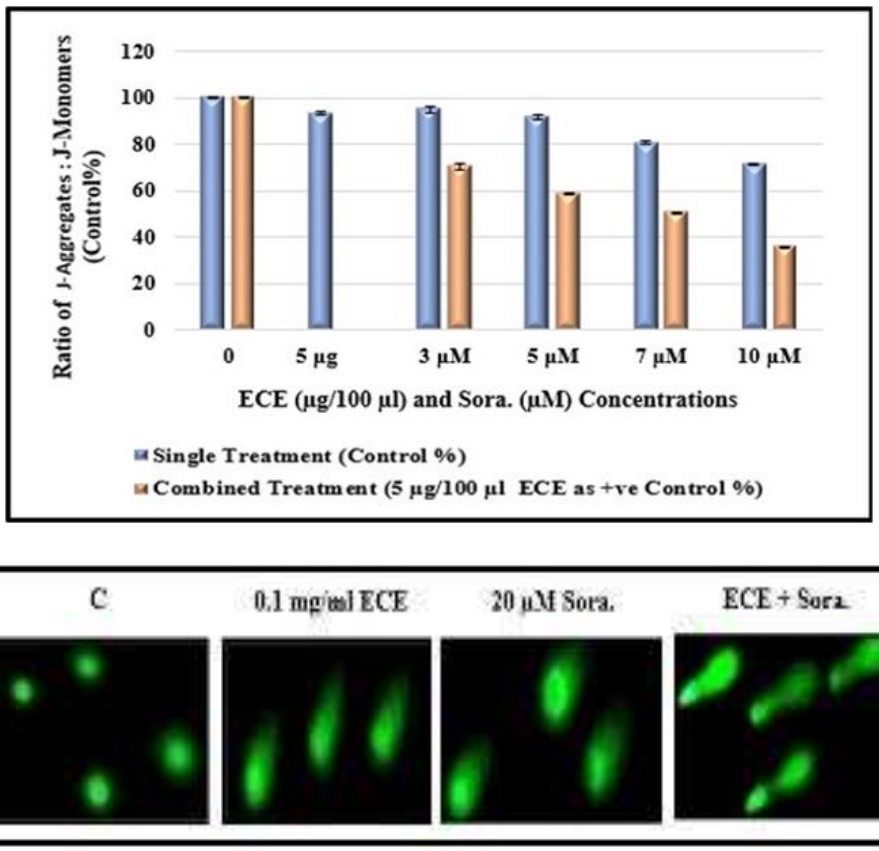


Figure 6. Apoptotic Comet formation by Single and Combined treatments of ECE and Sora. in HepG2 cells, there is clear appearance of Comet formation, which is a typical characteristic of apoptosis. Magnification x10.

The results (Figure 7.A) show that there was no DNA ladder formation in the samples after single treatment after 24 h. However, after 48 h ECE treatment and combined treatment (24 h), the results (Figure 7.B) showed that there was DNA ladder formation after the treatment.

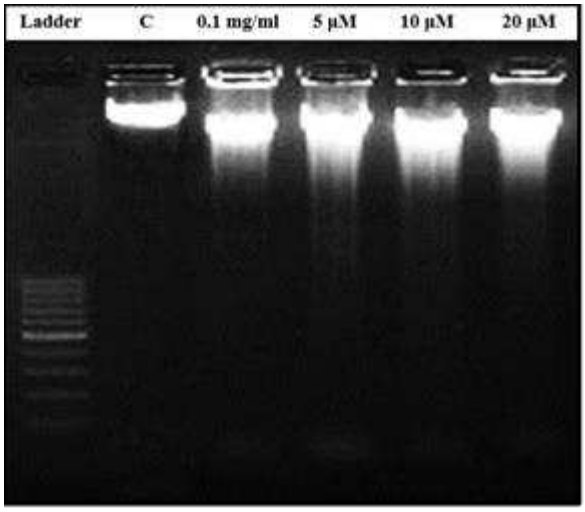


Figure 7. A. Detection of DNA ladder formation in apoptotic HepG2 cells induced by single treatment (0.1 mg/ml of ECE and 5, 10, 20 µM of Sorafenib) after 24 h. DNA ladder was not apparent in all doses of single treatment.

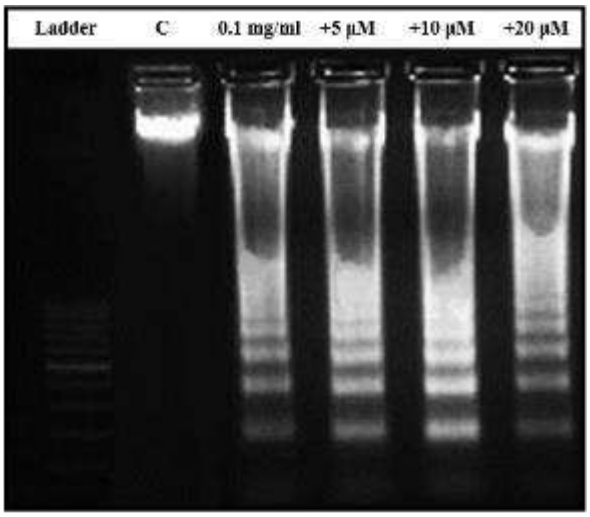


Figure 7. B. Detection of DNA ladder formation in apoptotic HepG2 cells induced by single dose of ECE (0.1 mg/ml) after 48 h and combined treatment (0.1 mg/ml of ECE with 5, 10, 20 µM of Sorafenib) after 24 h. DNA ladder was apparent.

DISCUSSION

Sorafenib displayed antiproliferative activity in liver cancer [8]. Some limitations of sorafenib use are its toxicity and adverse reactions [10]. Several research maintained that cardamom extracts can display anti-cancer activities, and anti-inflammatory, anti-proliferative and pro-apoptotic activities have been proposed as mechanisms underlying the anti-cancer properties of cardamom [36]. The present study was conducted to investigate the enhancement of sorafenib action by ECE.

Monoterpenes (the active ingredient in E. cardamom) are nonnutritive dietary components found in the plants. A number of these dietary monoterpenes have antitumor activity; α-terpineol (α-T) possesses a wide range of biological applications, one of which is the antiproliferative effect on human cancer cells [37, 38]. Hassan, Gali-Muhtasib [39] proved that α-T acts as a potential anticancer agent by suppressing nuclear factor-κB (NF-κB) signaling.

This study demonstrates that sorafenib inhibited cell proliferation and colony formation in a dose-dependent manner. The sorafenib concentration required to cause 50 % growth inhibition (IC50) after 24 h of incubation was 15 µM, which agrees with the results of Wei [40]. The IC_{50} of ECE after 24 h of incubation was 26 µg/100 µl,

which is in agreement with Zhihua [41]. The number of colonies created was further decreased by combined treatment compared to single treatments, suggesting an a synergistic effect on cancer cells when ECE is combined with sorafenib.

A wound healing assay was performed to confirm the results of the previous assays that were performed to evaluate the activities of the single treatment. Cell migration is a critical process for cancer cell spread, invasion, and distant metastasis [42]. The inhibition of cell migration and invasion in our study was a sign of the single treatment effectiveness. The wound healing assay results were in agreement with the previous published studies [43, 44]. In addition, this study evaluated the activities of the combined treatment (25 µg/ml of ECE with sorafenib) on cell migration, the width of the wounds appeared and expanded by the combined treatment more than single treatment. These results demonstrate that ECE may act as an anticancer agent against hepatic cancer cells.

ROS and the mitochondria play an effective function in encouraging the death of cells that occurs under both physiologic and pathologic circumstances [45]. The results (Figure 4) are in agreement with the results of Coriat [46] and Yadav [47]. The findings of this study show that the combined treatment (5 µg/100 µl ECE with Sorafenib) synergistically induced ROS-production in HepG2 cells.

ROS generation encourages the collapse of mitochondrial membrane breakdown, which leads to the release of MMP, one of the earliest intracellular causes of apoptosis [48]. The single treatment was observed to cause MMP emission, as assured by lowering of red fluorescence and an excess of green fluorescence of the JC-1 dye, which is in agreement with the results of Chu [49] and Itani [50]. In addition, this study found that the combined treatment (5 µg/100 µl ECE with sorafenib) synergistically induced mitochondrial damage in HepG2 cells more than single treatment did alone.

The extent of DNA damage was determined by using comet assay. The comet assay is almost passable as a classic marker in studying the apoptotic activity of anticancer factors and can differentiate the mode of cell death, whether apoptotic or necrotic [51]. Elkady [32] indicated that degradation of DNA into oligo nucleosomal fragments of multiples 180 base pairs is one of the necessary features of classic apoptotic cell death.

The results (Figure 7.A) showed that there was no DNA ladder after the treatment with the single treatment for 24 h, which is in agreement with Zhao [52]. In addition, our findings showed that the single ECE dose for 48 h and the combined treatment (0.1 mg/ml ECE with sorafenib) synergistically induced DNA ladder appearance (Figure 7.B) in HepG2 cells when treated for 24 h.

CONCLUSION

The data obtained demonstrated that the ECE strongly enhanced sorafenib-suppressed growth of HepG2 cells by induction of apoptotic cell death.

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