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Research article

Cytotoxic effect of *Ginkgo biloba* kernel extract on HCT116 and A2058 cancer cell lines

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ABSTRACT

While the pharmacology of *Ginkgo biloba* leaf extract has been studied extensively, little is known about the pharmacological potential of *Ginkgo biloba* seeds, although they contain similar active ingredients that are responsible for the therapeutic effects of the leaf extract. In this study we used 70%-methanol *Ginkgo biloba* kernel extract, quantified its bioactive constituents and tested their cytotoxic effect on two cancer cell lines, A2058 and HCT116, and the non-tumor cell line McCoy-Plovdiv. We studied the biological effect of the extract by real-time analysis in the xCELLigence system, WST-1 assay and LIVE/DEAD viability assay. We show that the extract significantly perturbed the viability of cancer cells in a concentration- and time-dependent manner. In contrast, non-cancerous McCoy-Plovdiv cells sustained their proliferation potential even at high concentrations of the extract. Therefore, we propose that the active constituents of the *Ginkgo biloba* endosperm extract may interact additively or synergistically to protect against cancer.

1. Introduction

Ginkgo biloba leaves and seeds have been used for centuries in traditional Chinese medicine. Nowadays *Ginkgo* leaf extract has stepped into the herbal spotlight as it has found a variety of therapeutic applications. The *Ginkgo* seed contains a kernel (nut), which is consumed as a delicious food in the Chinese, Japanese and Korean cuisine after fermentation, grilled or boiled but the medical significance of *Ginkgo* seeds has been somehow overlooked. The seeds are known to have a longer history of usage, being first mentioned in herbals in the Yuan dynasty, published in 1350 AD (Goh and Barlow, 2002). They have been used in China for treating pulmonary diseases such as asthma, coughs, and enuresis for several thousand years (Mahady, 2001) but solid research on their therapeutic effects is lacking.

As with any other seeds, the starch that must nourish the embryo during its development is a major constituent of *Ginkgo biloba* kernels; it accounts for 22% of kernel mass and ca. 50% of the dry matter (Spence and Jane, 1999). The content of lipids (3% of dry nut) and proteins (15% dry matter basis) is lower compared to other nuts (Duke, 1989). A few low molecular mass secondary metabolites extractable in organic solvents, namely methanol, have been also isolated from kernels. Most of them are identical to those isolated from leaves: flavonoids (quercetin, kaempferol and isorhamnetin in their glycosylated form or as aglycones) and terpenes (ginkgolides A, B, C and J, and bilobalide) (Zhou et al., 2014). Apart of this, the kernels also contain polyphenolic organic acids, carbohydrates, vitamins, inorganic salts and amino acids. Many of these have been shown to be beneficial for treating neurodegenerative diseases, cancer, cardiovascular diseases, stress responses, and mood and memory disorders (Nash and Shah, 2015).

Bioactive constituents extracted from *Ginkgo biloba* leaves such as flavonoids, their glycosides and terpene lactones, have attracted considerable attention in the therapy of Alzheimer's disease (Janßen et al., 2010; Müller et al., 2019; Singh et al., 2019; Zeng et al., 2017), cognitive disorders (Beck et al., 2016; Guan et al., 2018; Luo et al., 2018), cardiovascular disease (Li et al., 2019; Nash and Shah, 2015; Tian et al., 2017; Wu et al., 2019) and cancer (Bai et al., 2015; Liu et al., 2017; Park et al., 2016; Zhao et al., 2013). The pharmacology of individual constituents obtained from *Ginkgo biloba* leaves has been studied in preclinical and clinical trials

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(Canter and Ernst, 2007; Ji et al., 2020; Savaskan et al., 2018; Spiegel et al., 2018; von Gunten et al., 2016). Flavonoids and trilactone terpenes are believed to be responsible for most of the pharmacological properties of *Ginkgo biloba* leaf extracts, and it has been suggested that synergistic effects might be of importance. However, these experiments have been typically performed using unconjugated flavonoids (agycones) (Gibellini et al., 2011). Flavonoids are present in plants mainly as glycosides and the nature of the saccharide and position of glycosylation are important factors for their bioavailability (Hollman and Katan, 1997). Only limited data are available on the biological activity of the glycosylated flavonoids in *Ginkgo biloba* leaves. According to Feng et al. extracts enriched in aglycons have shown better anti-cancer activity compared to those rich in glycosylated flavonoids (Feng et al., 2009).

The other bioactive constituents of *Ginkgo biloba* leaf extracts, ginkgolides, have been clinically demonstrated to act as platelet-activating factor antagonists (Sun et al., 2015). In addition, bilobalides have shown anti-inflammatory properties in an animal model of stroke (Jiang et al., 2014a).

In contrast to the plenty of investigations on the pharmacology of the standardized *Ginkgo biloba* leaf extract EGb 761®, a limited number of studies have been conducted on the pharmacological potential of *Ginkgo biloba* exocarp extracts (Cao et al., 2017, 2019; Xu et al., 2003) and nuts. Only recently, a few reports have shed some light on the possible biological properties of *Ginkgo biloba* kernel extracts (Chassagne et al., 2019; Chen et al., 2002).

Generally, the pharmaceutical science is interested in the identification of individual compounds in plant extracts that possess valuable pharmacological properties because the knowledge about their therapeutic mechanisms is important to explain the pharmacology as a whole and the possible clinical applications of the extracts. Moreover, such natural compounds help in the design and development of new synthetic analogs (Koehn and Carter, 2005). On the other hand, the study of total plant extracts has some advantages. First, it provides initial information whether further separation and structural elucidation of prospective individual compounds makes sense. Secondly, traditional medicine uses total extracts in the form of macerates or infusions, or as in the case of *Ginkgo biloba* seeds the whole kernel, and from this point of view it is more important to know the biological properties of such extracts. Thirdly, sometimes the individual constituents, having differing mechanisms of action, may lead to a pharmacological synergy within the total extracts (Hristova et al., 2013).

In this study we used bioactive low-molecular mass compounds isolated from *Ginkgo biloba* kernels in 70% methanol, a solvent that is commonly used for extraction of both, polar and moderately polar molecules from plants, and tested the extracted compounds for their cytotoxic effect on two cancer cell lines, A2058 and HCT116, and the non-tumor cell line McCoy-Plovdiv.

2. Material and methods

2.1. Ginkgo biloba extract (EGB)

Mature seeds of *Ginkgo biloba* obtained from a tree located on the campus of the Medical College in Plovdiv, Bulgaria were harvested in November 2018. The seeds were shelled and the kernel (endosperm) was homogenized in a high-speed tissue homogenizer. Dry matter of kernels was determined gravimetrically according to Ph.Eu. 9.6 by heating at 105 °C to constant mass. Methanol Chromasolv® gradient grade for HPLC (Fisher Scientific, cat # 60-009-46) was used for extraction. The homogenized pulp (10 g) was dispersed in 70% methanol (1:10 w/v, dry matter base) and stirred continuously for 12 h at room temperature in a light-protected flask. After centrifugation at 6000 g for 10 min the supernatant obtained was filtered through Whatman 1 filter paper. The same procedure but with a 6-hour extraction was repeated twice on the residue. The three extracts were combined, and solvent was evaporated to dry residue at 30 °C under reduced pressure. The yield of methanol-soluble constituents was 8.28 ± 1.56% of *Ginkgo biloba* kernel dry matter (n = 3).

The dried extract was dissolved in 70% DMSO in water. Cells were treated with the following final concentrations of EGB in serum-free medium: 800 μg/ml; 750 μg/ml; 700 μg/ml; 650 μg/ml; 600 μg/ml; 500 μg/ml; 400 μg/ml; 300 μg/ml; 200 μg/ml. Control cells were treated with 0.8% DMSO in serum-free medium.

2.2. Chemical characterization by liquid chromatography with mass-detection (LC-MS)

LC-MS/MS analysis was performed on a Thermo Dionex Ultimate 3000 chromatographic system and a Thermo TSQ Quantum Access MAX triple quadrupole mass detector with a Heated Electrospray Ionization (HESI) ionizer. Separation of flavonoids was performed on a Syncronis C18 (150 × 4.6, 5 μm) chromatographic column in gradient mode with mobile phases A: 0.1% formic acid in acetonitrile-water (90:10, v/v) and B: 0.1% formic acid in acetonitrile-water (10:90, v/v) at a flow rate 0.7 ml/min. The gradient starts with 3% phase A and reaches 15% within 15 min, remains constant for 4 min and then returns to the starting conditions. HESI ionizer was used in negative mode, with spray voltage -3 kV, source temperature 350 °C, transfer capillary temperature 300 °C, sheath gas 50 AU, aux gas 5 AU.

The same chromatographic system was used for analysis of terpene trilactones but separation was carried out in isocratic mode with mobile phase 0.1% formic acid in acetonitrile-water (50:50, v/v) at a flow rate 0.5 ml/min.

Analysis of ginkgotoxin (4′-O-methylpyridoxine) was carried out on chromatographic column Hypersil C18 (150 × 3 mm, 3 μm) in isocratic

Table 1. Levels of bioactive constituents in 70% methanolic extract of *Ginkgo biloba* kernels. The results are presented per gram dry matter of the extract.

No	Compound	Transition	Amount (μg/g)
1	Quercetin	300.9 → 151.1	20.7
2	Kaempferol	285 → 185.1	55.9
3	Isorhamnetin	315 → 151	25.8
4	Rutin	609.2 → 300.3	8.85
5	Ginkgolide A	453 → 407	242
6	Ginkgolide B, J*	423 → 367.5	388
7	Ginkgolide C	439 → 383	143
8	Bilobalide	324.9 → 163	122
9	Ginkgotoxin	183.97 →152.04	335

* Ginkgolide B and ginkgolide J are isomers and could not be separated upon the chromatographic conditions used.

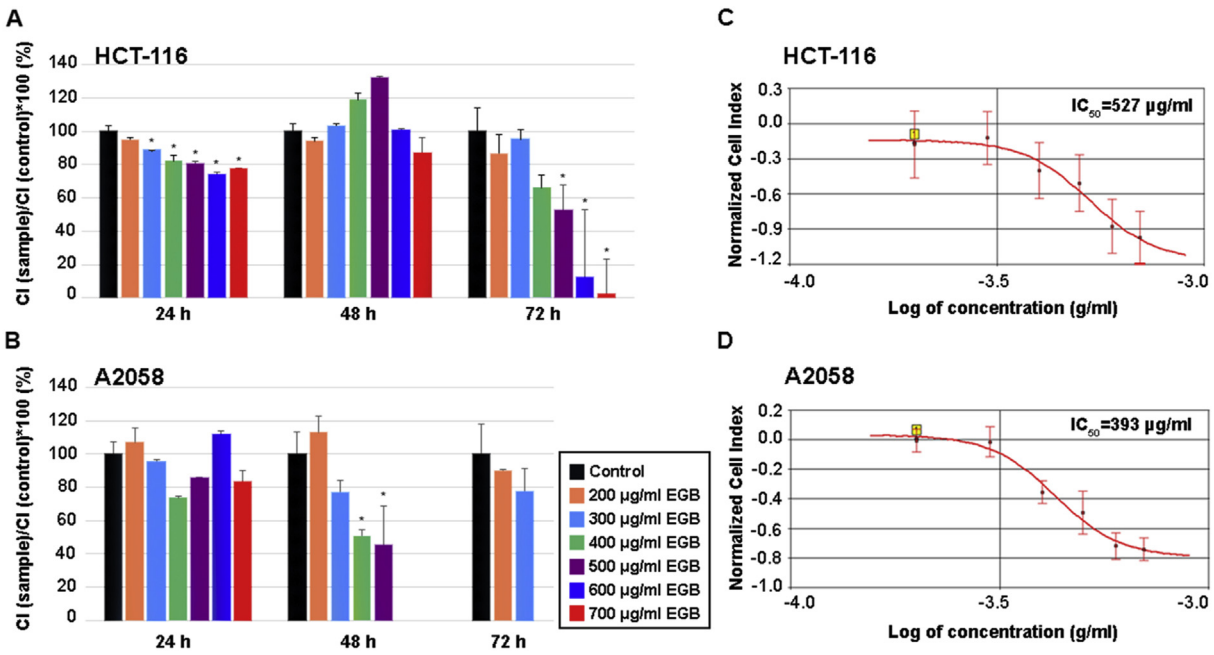

Figure 1. Real-time analysis of HCT116 and A2058 cells treated with total endosperm EGB extract. Cells were cultured in E-plates, placed in the xCELLigence system in the cell culture incubator and incubated for 24 h. After that the plates were taken out and the growth medium in the wells was replaced with growth medium, containing different concentrations of EGB. Cells treated with 0.8% DMSO in serum-free medium served as controls. Graphs are generated in Excel (A, B) and in the RTCA Software 2.0 (C,D). A, B: The graphs show the dynamics of the cell index at three selected timepoints of EGB treatment, 24 h, 48 h and 72 h. The cell index of the treated cells is normalized to that of the control cells at the respective timepoint multiplied by 100. The cell indices of A2058 cells treated with concentrations above 600 µg/ml (B, 48 h) and above 400 µg/ml (B, 72 h) have values below 0, which means that there are no cells attached to the surface, so the viability is considered 0% and bars are thus missing in the graph. Asterisks in the graphs mark the concentrations of EGB at which statistically significant ($p < 0.05$) changes in the cell index occur, as determined by one-way ANOVA analysis of the data. For A2058 cells (B) such occur for all concentrations above 400 µg/ml at 48 and 72 h. C, D: Sigmoidal dose-response curves derived with the data analysis tool of the RTCA 2.0 software. The curves were used to determine IC_{50} values for both cell lines. The IC_{50} value for HCT116 cells is estimated to be 527 µg/ml and for A2058 cells – 393 µg/ml.

mode with mobile phase 0.1% formic acid in acetonitrile-water (60:40, v/v) at a flow rate 0.5 ml/min.

A set of commercial standards produced by Merck-Sigma-Aldrich was used to quantify the target compounds: *Ginkgo* Biloba Flavonoids Mix (cat # G-014), Rutin trihydrate (cat # 78095), *Ginkgo* Biloba Terpene Lactones Mix (cat # G-013) and ginkgotoxin (cat # 89960).

The total concentrations of polyphenols and flavonoids in the extract were determined according to European Pharmacopeia (European pharmacopoeia 8.0, vol. 1, p. 275. Council of Europe, Strasbourg) and the method proposed by Morado-Castillo et al. (2016) respectively. Total carbohydrate content was determined according to the anthrone/sulfuric acid method (Haldar et al., 2017). Qualitative and quantitative determinations of phenolic acids were performed by HPLC system with UV-detection as previously described (Marchev et al., 2011).

2.3. Cell culture

Human melanoma cells A2058 (ATCC: CRL-11147) and colon carcinoma cells HCT116 (ATCC: CCL-247) were cultured in DMEM growth medium (PAN Biotech, cat # P04-04500), supplemented with 10% FCS (PAN Biotech, cat # P30-1501), 100 IU penicillin and 100 µg/ml streptomycin (PAN Biotech, cat # P06-07100). Serum-free fibroblast cells McCoy-Plovdiv were cultured in DMEM/F12 (1:1) growth medium (PAN Biotech, cat # P04-41150) according to Draganov et al. (2005). All cell cultures were grown in a cell culture incubator at 37 °C, 5% CO_2 and high humidity.

2.4. LIVE/DEAD viability assay

Cell cultures were trypsinized with 0.25% trypsin/0.02% EDTA in PBS (PAN Biotech, cat # P10-020100) and the number of cells was measured in a Neubauer counting chamber. The single cell suspension was brought to a concentration of $2x10^5$ cells/ml. 200 µl of the cell suspension were seeded in each well of a 96-well plate and the plate was incubated in an incubator for 24 h. Afterwards the growth medium was removed and replaced with serum-free medium, containing EGB in different concentrations. Control cells were treated with 0.8% DMSO in serum-free medium. Dead and living cells were studied every 24 h using staining with 2 µM calcein-AM and 4 µM ethidium-homodimer 1 in PBS following the protocol of the manufacturer (ThermoFisher Scientific, cat # L3224). Staining with calcein-AM shows active esterase activity and is thus indicative of living cells. Positive staining with the ethidium-homodimer 1 shows loss of membrane integrity, a characteristic feature of dead cells. Cell vitality was evaluated microscopically using an inverted microscope Nikon Eclipse TS100. Images were prepared in Adobe Photoshop CS5.

2.5. Electric impedance measurement

We used the xCELLigence RTCA-DP system (ACEA Biosciences) for real-time analysis of the studied cell lines. The xCELLigence system measures the electric impedance – a physical quantity dependent on the number of cells attached to the surface of the plate well. Thus, the derived metric called the cell index is proportional to the number of living cells. 200 µl of HCT116 and A2058 cells were seeded at a concentration of $2x10^5$ cells/ml in the wells of an E-Plate VIEW 16 (ACEA Biosciences, cat # 300600880). Cells were cultured in the real-time cell analyzer in a cell

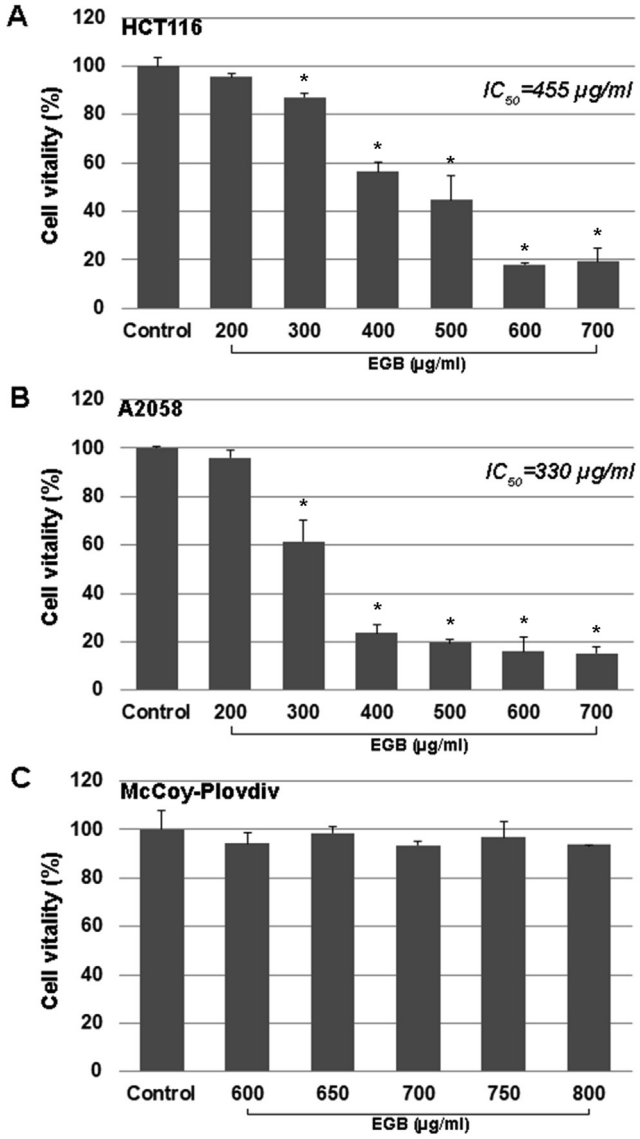

Figure 2. WST-1 assay for cell vitality of HCT116 (A), A2058 (B) and McCoy-Plovdiv (C) cells. WST-1 colorimetric assay was used to determine the number of living cells upon treatment with increasing concentrations of total EGB extract. Control cells were treated with 0.8% DMSO. Results were analyzed and graphs were prepared in Excel. Asterisks in the graphs mark the concentrations of EGB at which statistically significant ($p < 0.05$) changes in cell vitality occur, as determined by one-way ANOVA analysis of the data.

culture incubator at 37 °C and 5% CO_2. After 24 h the plates were taken out of the incubator and the growth medium was replaced by a growth medium containing EGB in the selected concentrations. Control cells were treated with 0.8% DMSO in serum-free medium. Graphs were generated and results were analyzed in the RTCA 2.0 software and Excel. Cells were observed under an inverted microscope every 24 h. After 72 h of treatment cell vitality was measured with WST-1 (Abcam, cat # ab155902).

2.6. Cytotoxicity assay

After 72-hour treatment with EGB cell vitality was measured quantitatively using the reagent WST-1. Cells were incubated with the reagent for 4 h at 37 °C. The absorption of the color product was measured at a 450/620 nm wavelength with an ELISA reader *Sunrise* (Tecan, Maennedorf, Switzerland). Each EGB concentration was tested in duplicate. Results were analyzed and graphs were prepared in Excel. The optical density (OD %) value was calculated with the following formula: Absorption (treated cells)/Absorption (control cells)*100. Error bars in the graphs represent the coefficient of variance (CV), calculated as the proportion of the standard deviation from the mean absorption value of two measurements multiplied by 100.

2.7. Statistical analysis

Statistical calculations were performed using SPSS v.27. Data were considered significant at $p < 0.05$. A two-way ANOVA followed by a Tukey HSD post-hoc test was conducted on the real-time data to evaluate the effects of concentration and exposure time as well as their interaction effects on cell viability. In turn, the WST-1 data were analyzed using a one-way ANOVA test.

3. Results

The chosen chromatographic conditions allowed a good separation and quantification of ten constituents of the 70% methanolic extract of *Ginkgo biloba* kernels known to be responsible for the characteristic therapeutic properties of leaf extract (Table 1). Chromatograms of standards and analytes are available as supplementary material (Supplementary Figures 1–4).

Among 11 phenolic acids searched (gallic, 3,4-dihydroxybenzoic, salicylic, chlorogenic, vanillic, caffeic, syringic, p-coumaric, sinapinic and ferulic), only p-coumaric acid was detected in the extract and its amount was 478 µg/g.

Determination of total polyphenol, flavonoid and carbohydrate content using spectrophotometric methods showed that 1 g dry matter of the extract contains 118 ± 3 mg gallic acid equivalents, 19.6 ± 0.8 mg quercetin equivalents and 375 ± 6 mg glucose equivalents.

To study the potential cytotoxic effect of the total *Ginkgo biloba* endosperm extract (EGB) we applied three different methods – real-time analysis in the xCELLigence system, WST-1 colorimetric assay and microscopic LIVE/DEAD viability assay. We treated HCT116 and A2058 cancer cells with the following concentrations of EGB: 700, 600, 500, 400, 300 and 200 µg/ml. In addition, we studied the effects of EGB on the non-cancerous fibroblast cell line McCoy-Plovdiv.

The impedance measurement and the two-way ANOVA test on the real-time data revealed that the effects of concentration and exposure time on both HCT-116 and A2058 cell viability, as well as their interaction effect, are statistically significant. The cell index for both cancer cell lines drops dramatically when cells are treated with the highest concentrations of EGB, 700 and 600 µg/ml. For the HCT116 cell line this occurs at 24 h of treatment, when cell vitality drops to below 80% (Figure 1A, Supplementary figure 5A). Interestingly, even cells treated with the highest concentrations of the extract seem to overcome the cytotoxic effect because at 48 h the cell index of all treated cells increases to approximately the same or even higher level than that of control untreated cells. However, a very strong cytotoxic effect is manifested for all extract concentrations, with exception of 200 and 300 µg/ml, at 72 h of treatment of HCT116 cells. By that time the cell index drops by 34–47% for cells treated with 400 and 500 µg/ml and by 88–98% for cells treated with 600 and 700 µg/ml of the extract. The effect of EGB is much stronger and occurs earlier in the A2058 cell line. Already at 48 h of treatment, the cell index of cells treated with intermediate concentrations, 400 and 500 µg/ml, drops by half, whereas for the two highest concentrations it goes below zero (Figure 1B, Supplementary figure 5B). At 72 h only those wells treated with the lowest concentrations of EGB seem to contain viable cells since the cell index is either close to or above 80% compared to the control. Despite the different behavior of the two cancer cell lines, the real-time analysis clearly demonstrated a gradual decrease of the cell index with higher concentrations of the extract (Figure 1, Supplementary Figure 5). The dose-response curve generated in the RTCA software

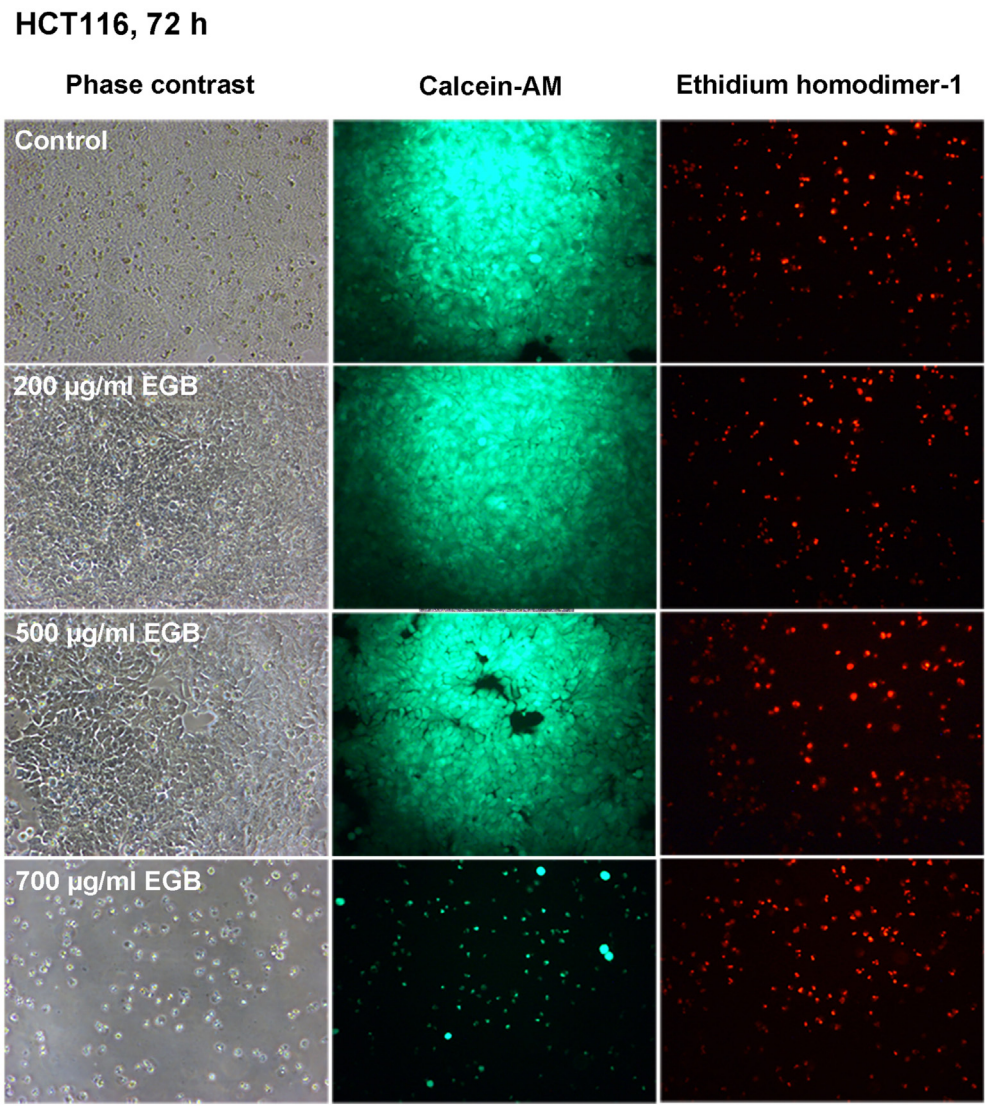

Figure 3. LIVE/DEAD viability assay for HCT116 cells. Staining with calcein-AM and ethidium homodimer-1 was used to display living and dead cells, respectively, upon treatment with different concentrations of EGB. Images are taken after 72 h of treatment and three representative concentrations are shown – 200, 500 and 700 µg/ml EGB, as well as control non-treated cells. For images of the other two time points – 24 and 48 h, please see Supplementary Figure 6 A1, 2. Magnification: 200X.

showed an IC_{50} value of 527 µg/ml for HCT116 cells (Figure 1C) and 393 µg/ml for A2058 cells (Figure 1D).

At the end of the real-time experiment cell vitality was also measured with the WST-1 reagent (Figure 2A,B). The results confirm a strong cytotoxic effect on the two cancer cell lines with IC_{50} values of 455 µg/ml for HCT116 cells (Figure 2A) and 330 µg/ml for A2058 cells (Figure 2B). Thus, the xCELLigence and WST-1 assays demonstrated similar overall trends with a very strong correlation between the two sets of data for both HCT116 ($r(12) = 0.950$, $p < 0.001$) and A2058 ($r(12) = -0.926$, $p < 0.001$) cell lines.

It is worth noting that the number of living cells in the WST-1 test does not fall below 20% even for the highest concentrations and for the more sensitive cell line A2058. Thus, decreased cell attachment that is indicated by the cell index value in the xCELLigence analysis does not necessarily mean that cells are metabolically dead. Indeed, this was confirmed by the microscopic evaluation using the LIVE/DEAD cell vitality assay. The two cancer cell lines were treated with the same concentrations of the EGB extract, stained with the LIVE/DEAD cell vitality assay every 24 h of treatment and observed under the microscope. After 72 h in wells of both HCT116 and A2058 cells treated with 700 µg/ml EGB the monolayer is clearly disrupted and the number of living cells is much lower than in the control wells or those with cells treated with lower EGB concentrations (Figures 3 and 4). The HCT116 cell monolayer seems better preserved at 500 µg/ml EGB (Figure 3) in comparison to the A2058 monolayer (Figure 4), although the number of dead cells as visualized by staining with ethidium-homodimer was quite high for both cell lines. Naturally, after 72 h of treatment a lot of dead cells were also observed at low EGB concentrations and in the control cell wells but in those wells the number of living cells was also much higher and the monolayer was sustained (Figures 3 and 4). In agreement with the results of the WST-1 assay and despite the altered morphology of cells and their visible rounding and detachment from the bottom of the wells, there are still viable cells in the wells treated with the highest EGB concentration. Thus, the combination of methods we chose for our study is suitable to better understand the behavior of cells and the kinetics and specifics of their response to EGB treatment.

To check whether our EGB extract exerts a specific anti-tumorigenic effect, we treated non-cancerous fibroblast McCoy-Plovdiv cells with the same concentrations of the extract and performed LIVE/DEAD cell staining (Figure 5). We did not observe a strong cytotoxic effect on those cells after 72 h of treatment even at the highest used concentration (Figure 5). Nevertheless, the LIVE/DEAD staining results suggested that

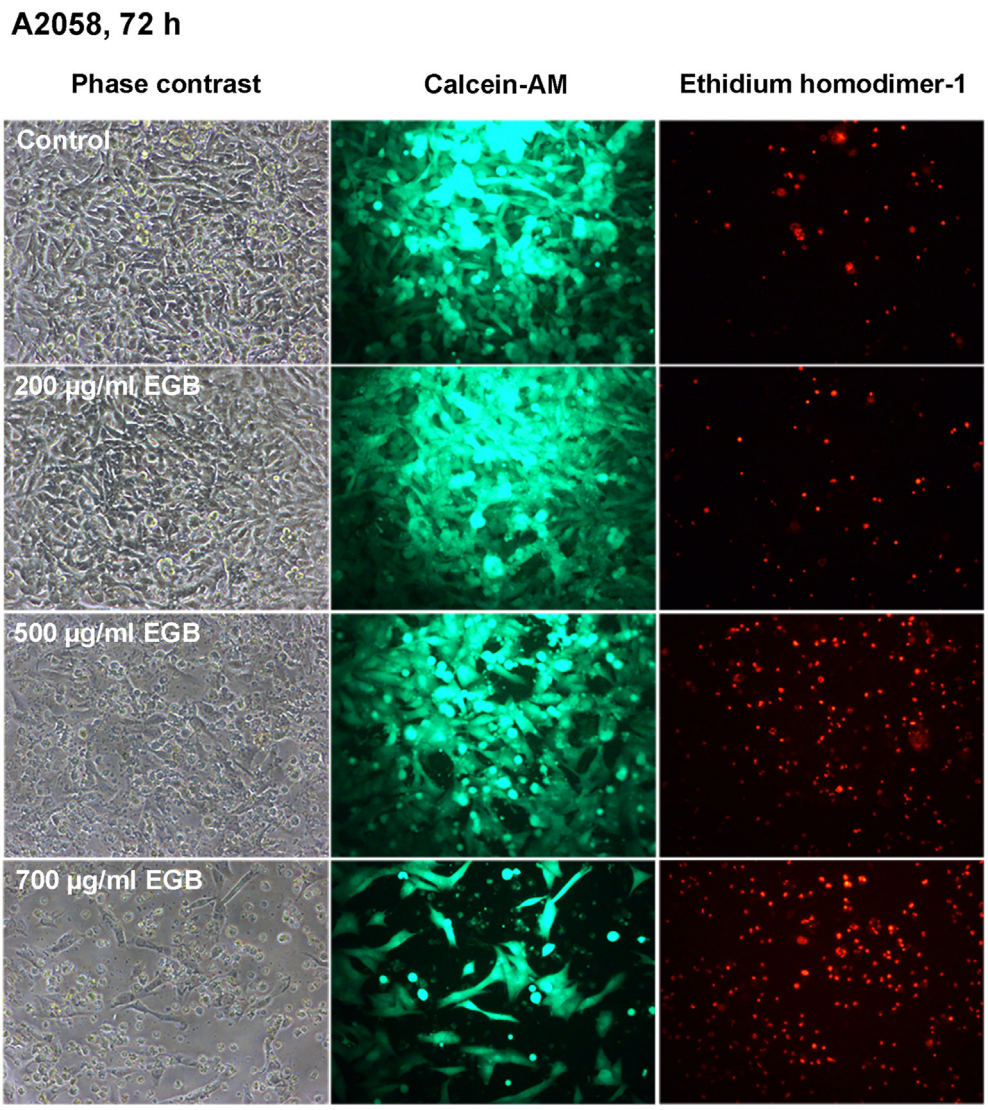

Figure 4. LIVE/DEAD viability assay for A2058 cells. Staining with calcein-AM and ethidium homodimer-1 was used to display living and dead cells, respectively, upon treatment with different concentrations of EGB. Images are taken after 72 h of treatment and three representative concentrations are shown – 200, 500 and 700 μg/ml EGB, as well as control non-treated cells. For images of the other two time points – 24 and 48 h, please see Supplementary Figure 6 B1, 2. Magnification: 200X.

the cytotoxic effect of EGB, if any, could be manifested only at very high concentrations. To test this, we treated the McCoy-Plovdiv cells with 5 concentrations of EGB – 800, 750, 700, 650 and 600 μg/ml, and measured cell vitality with WST-1 after 72 h (Figure 2C). We found that none of the tested concentrations significantly reduced the viability of cells. Moreover, in wells with the highest tested EGB concentration the number of living cells decreased by only 7% compared to the control.

4. Discussion

A number of flavonoids have been shown to suppress carcinogenesis in various clinical studies (Romagnolo and Selmin, 2012). The good antioxidant properties of these natural products were the first mechanism of action proposed. In fact, flavonoids are highly effective scavengers of most types of reactive oxygen species, which are possibly involved in DNA damage and tumor promotion (Cerutti, 1985). Nevertheless, dietary antioxidants have consistently failed to reduce the incidence of carcinoma in prospective human clinical trials (Chandel and Tuveson, 2014). In recent years, experimental animal studies have provided growing evidence for the beneficial action of some flavonoids (quercetin, kaempferol, galangin, and apigenin) on multiple cancer-related biological pathways: carcinogen bioactivation, cell-signaling, cell cycle regulation, angiogenesis, and inflammation (reviewed by (Le Marchand, 2002)).

In their work Zhou et al. (2014) have identified 26 compounds in 70% methanol extract of *Ginkgo biloba* kernels. Our data confirmed their conclusion that terpene trilactones that are unique for this species were dominant components since they were present in the extract in much higher concentrations than free flavonoids. However, it should be pointed out that the reported data are for free aglycones, whereas in plants flavonoids exist predominantly in the form of glycosides. Comparing the data from the chromatographic and spectrometric methods, it can be speculated that about 95.5% of the flavonoids in the *Ginkgo biloba* extract are glycosylated to one degree or another.

Ginkgotoxin is known as antivitamin B_6 that possibly causes neuronal symptoms (Jang et al., 2015). The presence of ginkotoxin has not been reported by Zhou et al. but its concentration in our methanolic extract was surprisingly high.

The results of our study clearly demonstrate that the total *Ginkgo biloba* kernel extract causes induced cell death of HCT116 and A2058 cancer cells. This effect is dose-dependent as shown by the xCELLigence analysis and the WST-1 assay, and time-dependent as confirmed by the

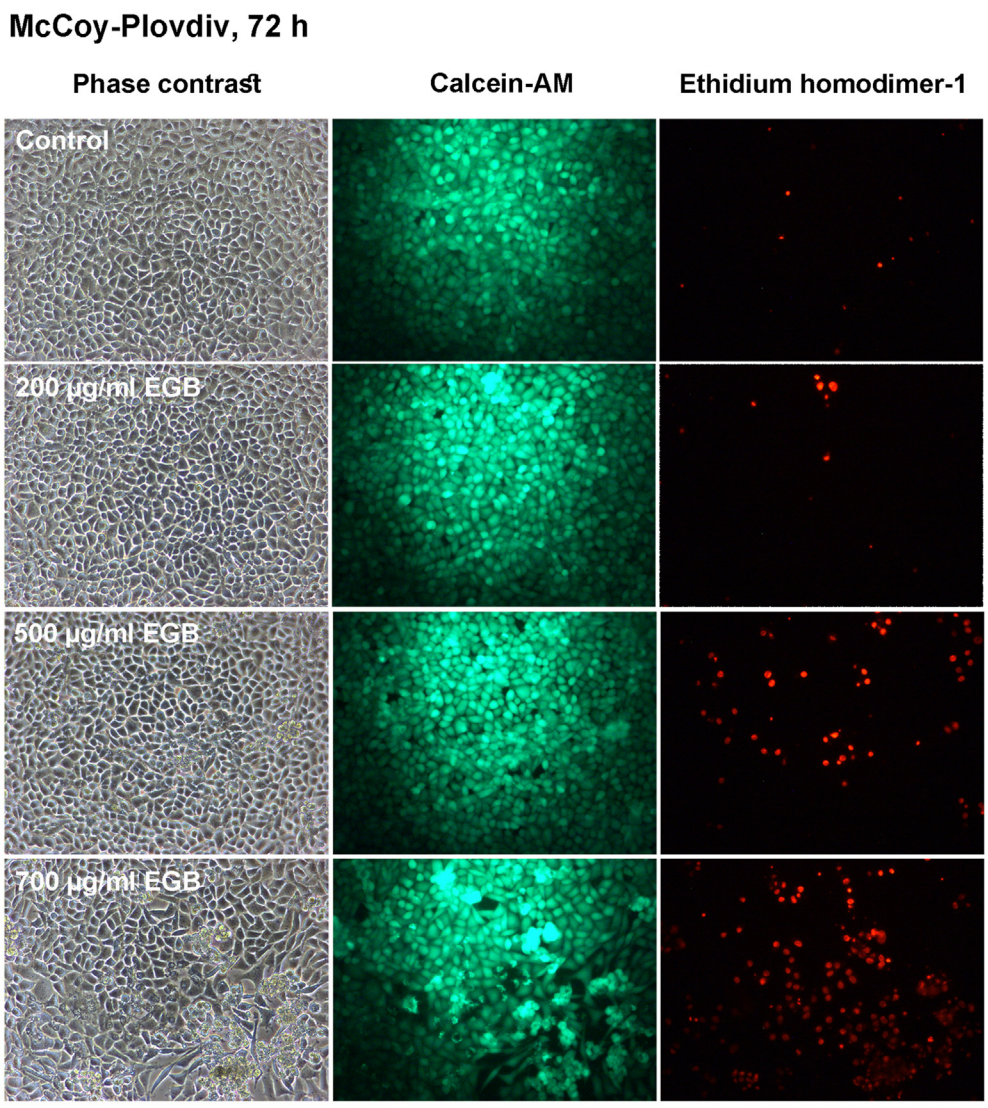

Figure 5. LIVE/DEAD viability assay for McCoy-Plovdiv cells. Staining with calcein-AM and ethidium homodimer-1 was used to display living and dead cells, respectively, upon treatment with different concentrations of EGB. Images are taken after 72 h of treatment and three representative concentrations are shown – 200, 500 and 700 µg/ml EGB, as well as control non-treated cells. For images of the other two time points – 24 and 48 h, please see Supplementary Figure 6 C1, 2. Magnification: 200X.

real-time analysis and the microscopic evaluation with the LIVE/DEAD cell vitality assay. The comparison of the IC_{50} values calculated for the two cell lines indicated a higher sensitivity of the melanoma cells to the components of the extract. Whereas the number of living cells decreased to 20% only at the two highest concentrations of the EGB extract in the case of the colorectal cancer cell line HCT116, it dropped to this level already at 400 µg/ml of EGB for the melanoma cells A2058. Thus, we show that EGB exerts strong but differential cytotoxic effect on different types of cancer cells.

Our observations using the LIVE/DEAD assay show that within 24 h of treatment the cells do not undergo any major changes in terms of morphology, vitality and the state of the cell monolayer. This implies that the active components of the extract do not cause a direct cytotoxic effect by attacking the cell membrane or other cell compartments important for cell homeostasis and vitality. In contrast, the destruction of the cell monolayer becomes very prominent at the 48th, and especially at the 72nd hour of treatment, as demonstrated by both LIVE/DEAD staining and real-time analysis, which is indicative of apoptosis. An apoptotic effect of polysaccharides isolated from a *Ginkgo* kernel water extract has been previously demonstrated for the human hepatocellular carcinoma cell line SMMC-7721 (Chen et al., 2002). However, in our study the concentrations of the total endocarp extract that induced cell death are almost a thousand times lower. The observed apoptotic effects can be caused either by activation of cellular mechanisms inducing cell death or by inhibition of important signaling pathways and mechanisms that ensure cell survival and vitality. The two hypotheses are not mutually exclusive, but the fine cell and molecular mechanisms that lead to them are beyond the scope of the work presented here and remain to be investigated in more detail in the future.

It is worth noting that EGB did not exert any inhibitory or cytotoxic effect on the serum-free McCoy-Plovdiv cell line, which was previously shown to be significantly more sensitive to different chemicals than its serum equivalent (Docheva et al., 1999). The cell line has been thus repeatedly used to study the biological activity of various substances, e.g. *Folia betulae* dry extract (Penkov et al., 2015), indomethacin-loaded nanoparticles (Andonova et al., 2015), antinuclear and anti-mitochondrial antibodies (Fransazov et al., 2008; Zagorov et al., 2007). Still, even in the non-cancerous cell line the number of dead cells is higher in the wells treated with high EGB concentrations than in the control wells. This can be explained with an early and short-term cytotoxic effect of EGB occurring before the 24th hour of treatment, which, however, does not perturb the proliferative potential of the cells and they continue to grow normally after overcoming the initial stress. In support of this notion, the cell monolayer is preserved in all treated cells at all

tested time-points (Figure 5, Supplementary figure 6C) and the number of living cells stained in green is comparable to that in the control wells. A drawback of our study is the missing real-time analysis of McCoy-Plovdiv cells that would possibly confirm our hypothesis. Unfortunately, we could not perform this analysis because McCoy-Plovdiv cells do not adhere properly to the surface of the E-plates and until now we have not identified the appropriate conditions to improve their adhesion behavior. Collectively, our results on McCoy-Plovdiv cells suggest that EGB exhibits cancer-cell specific cytotoxic effects by yet unknown mechanisms.

A plethora of research has focused on the properties of *Ginkgo biloba* leaf extracts including their anti-cancer effects. Attempts to clarify the mechanisms of cytotoxic action have been made primarily with the well characterized commercially available leaf extract EGb 761®. It has been shown that the extract causes cell death of hepatocarcinoma cells by inhibiting the expression of PCNA and activating p53 driving apoptosis (Chao and Chu, 2004), as well as by regulating the PI3K/Akt/mTOR signaling pathway and its downstream targets eIF4 and p70SK6, thus impairing protein synthesis and perturbing cell growth (Czauderna et al., 2018). EGb 761® inhibits the proliferation of breast, glioma and hepatocarcinoma cell lines by downregulation of the mRNA levels of the peripheral-type benzodiazepine receptor, a cholesterol-binding protein with a role in various processes, including apoptosis (Pretner et al., 2006). A recent study has demonstrated that treatment with *the Ginkgo biloba* leaf extract suppresses the metastatic potential of colon cancer cells by inducing the expression of lincRNA-p21 that stabilizes E-cadherin (Chang et al., 2018). An additional anti-cancer effect of EGb 761® has been demonstrated in gastric cancer cells, whose sensitivity to chemotherapy was increased upon treatment with the extract via suppression of the KSR1-mediated ERK1/2 pathway (Liu et al., 2015). Ginkgolide B, a major constituent of *Ginkgo* extracts, has been also shown to sensitize ovarian cancer calls to cisplatin and induce apoptosis in these cells by upregulation of p21 and p27, downregulation of cyclin D and activation of caspases 8 and 3 (Jiang et al., 2014b). Ginkgolic acids, found in *Ginkgo biloba* crude extracts, inhibit the migration of breast cancer cells by inhibiting NEMO-sumoylation and NF-κB activity (Hamdoun and Efferth, 2017) or by inactivation of the PI3K/Akt/mTOR signaling pathway in lung cancer cells (Baek et al., 2017). The apoptotic effect of gikgolic acids involves decreased expression of the anti-apoptotic factor Bcl-2 and increased expression of the pro-apoptotic Bax (Zhou et al., 2010). In addition to their anti-cancer effects, *Ginkgo* extracts have been suggested to possess genotoxic activity inducing DNA damage in mouse lymphoma cells (Lin et al., 2014) and in human hepatoma cells (Zhang et al., 2015), the latter being linked to topoisomerase-II inhibition by the *Ginkgo biloba* extract. In contrast, a study on human hepatoma HepG2 cells has not noticed any effects of *Ginkgo biloba* on DNA damage or carcinogenesis-related gene expression (Grollino et al., 2017). Thus, the various effects of *Ginkgo biloba* leaf extracts on cell survival may vary depending on the cell line, cell morphology (normal *vs.* malignant), and the dosage of the extract or the individual constituents. In addition to the leaf extracts, *Ginkgo biloba* exocarp extract has been also shown to exhibit anti-cancer properties, e.g. by inhibiting the proliferation and migration of B16–F10 melanoma cells via the PI3K/Akt/NF-κB/MMP-9 pathway (Cao et al., 2018).

Although the seeds have been long used in traditional Chinese medicine, much fewer studies exist describing their biological effects and therapeutic profile. Recently, Chassagne et al. (2019) demonstrated the antimicrobial activity of *Ginkgo* seed extracts, including kernel, against skin pathogens but, to our knowledge, our study is the first to show the anti-cancer properties of a total *Ginkgo biloba* kernel extract.

5. Conclusion

The presence of a strong cytotoxic effect of the *Ginkgo biloba* kernel extract on cancer cells was unequivocally demonstrated and confirmed by all the used methods. The extract significantly perturbed the proliferation and vitality of two cancer cell lines, HCT116 and A2058, whereas no such effect was observed for the non-tumor cell line McCoy-Plovdiv. Therefore, we propose that the *Ginkgo biloba* endosperm extract has anti-cancer properties, whose potential should be explored for the development of novel medicines.

Declarations

Author contribution statement

Y. Feodorova: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

T. Tomova: Performed the experiments; Analyzed and interpreted the data.

D. Minchev: Analyzed and interpreted the data.

V. Turiyski: Contributed reagents, materials, analysis tools or data.

M. Draganov: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

M. Argirova: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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