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Cytotoxicity, genotoxicity and gene expression changes elicited by exposure of human hepatic cells to Ginkgo biloba leaf extract

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Abbreviations:

AFB1, Aflatoxin B1; CAR, constitutive nuclear androstane receptor; CARKO, CAR-Knockout; DMSO, Dimethyl Sulfoxide; ECACC, European Collection of Cell Cultures; EMA, Committee on Herbal Medicinal Products of the European Medicine Agency; IARC, International Agency for Research on Cancer; ISO, International Organization for Standardization; JaCVAM, Japanese Center for the Validation of Alternative Methods; NTP, National Toxicology Program; OECD, The Organisation for Economic Co-operation and Development; PBS, Phosphate Buffered Saline; ROS, Reactive Oxygen Species.

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Abstract

The use of Ginkgo biloba leaf extract as nutraceutical is becoming increasingly common. As a consequence, the definition of a reliable toxicological profile is a priority for its safe utilization. Recently, contrasting data have been reported on the carcinogenic potential of Ginkgo biloba extract in rodent liver. We measured viability, Reactive Oxygen Species (ROS), apoptosis, colony-forming efficiency, genotoxicity by comet assay, and gene expression changes associated with hepato-carcinogenicity in human cells of hepatic origin (HepG2 and THLE-2) treated with different concentrations (0.0005-1.2 mg/mL) of Ginkgoselect®Plus*. Our analyses highlighted a decrease of cell viability, not due to apoptosis, after treatment with high doses of the extract, which was likely due to ROS generation by a chemical reaction between extract polyphenols and some components of the culture medium. Comet assay did not detect genotoxic effect at any extract concentration. Finally, the array analysis detected a slight decrease in the expression of only one gene (IGFBP3) in Ginkgo-treated THLE-2 cells as opposed to changes in 28 genes in Aflatoxin B1 treated-cells. In conclusion, our results did not detect any significant genotoxic or biologically relevant cytotoxic effects and gross changes in gene expression using the Ginkgo extract in the hepatic cells tested.

*Ginkgoselect® is a registered trademark of Indena S.p.A., Italy

Keywords:

1. Introduction

The use of plant-derived bioactive compounds as dietary supplements has dramatically increased in recent years. Within this context, the evaluation of the efficacy, quality and toxic potential of the natural products represents a central issue for human health (Izzo et al., 2016).

Ginkgo biloba leaf extract is one of the most extensively used phytopharmaceutical drugs. The extract displays several pharmacological activities, mostly associated with the presence of various flavonoid and terpenoid components, that include antioxidants, radical scavengers, antiapoptotic molecules and inhibitors of platelet aggregation and inflammation (Pereira et al., 2013; Diamond et al., 2013; Serrano-García et al., 2013).

Due to these characteristics, Ginkgo biloba has been reported to have therapeutic effects in the treatment of cardiovascular, cerebrovascular and neurologic diseases (Gauthier et al., 2014; Nash and Shah, 2015) and to be safe and well tolerated for human consumption (Heinonen and Gaus, 2015).

Recently, carcinogenicity studies in rodents provided evidence of carcinogenic risk associated with increased incidence of hepatocellular carcinoma and hepatoblastoma in animals fed with high doses of Ginkgo biloba extract (NTP, 2013; Rider et al., 2014). Following the National Toxicology Program (NTP) report (NTP, 2013), the Ginkgo biloba extract has been classified as possibly carcinogenic in humans and included in the Group 2B by the International Agency for Research on Cancer (IARC, 2016). However, the conclusions of the NTP study have not been unanimously accepted (Heinonen and Gaus, 2015; Gaus, 2014; Kissling et al., 2015). In addition, the Committee on Herbal Medicinal Products of the European Medicine Agency (EMA) has issued documents stating that, at

present, there is no proof for an increased cancer risk in patients taking Ginkgo products at their approved posology (EMA, 2014).

Maeda and others (Maeda et al., 2015) investigated the involvement of the constitutive nuclear androstane receptor (CAR) using CAR-Knockout (CARKO) mice fed with the same Ginkgo biloba extract used in NTP experiments. Increased hepatocellular proliferation and adenomas were induced in wild type but not in CARKO mice, strongly suggesting the involvement of CAR in Ginkgo extract induced carcinogenicity. CAR activation leading to cell proliferation is a well known carcinogenic mechanism in rodents (LeBaron et al., 2014); however, the same mechanism is not deemed relevant for human hepatocytes since in these cells CAR activators induce drug metabolizing enzymes, but not proliferation (Elcombe et al., 2014; Lake et al., 2014). Thus, although flavonoids in the Ginkgo extract were shown to activate CAR and to induce downstream metabolizing enzyme expression in human hepatocyte cell lines (Li et al., 2009), such a response should not have consequences on human cancer risk.

Studies aiming to evalute genotoxicity in vitro and in vivo would be useful to clarify the mechanisms behind rodent liver tumors and to exclude possibile genotoxic effects in humans. Until now, few studies have investigated the extract as a whole. DNA damage was detected by comet assay in mouse lymphoma cells (Lin et al., 2014) and in human hepatoma cells (Zhang et al., 2015), where damage was linked to topoisomerase-II inhibition. DNA damage repair activation was demonstrated by increase of γH2AX and phosphorylation of Chk1 and Chk2 (Lin et al., 2014; Zhang et al., 2015). Notably, in the study on hepatoma cells not all tested extracts yielded positive results. Gene mutation induction was shown in mouse lymphoma cells, with loss of heterozygosity in the mutants suggesting chromosome damage (Lin et al., 2014). However, neither DNA damage and

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gene mutations in liver, nor micronuclei in bone marrow appeared to be increased in mice exposed to a Ginkgo biloba extract up to 2000 mg/kg bw/day (Maeda et al., 2014). More data are available on the genotoxicity of specific constituents of the extract, with quercetin (Resende et al., 2012; Caria et al., 1995; Lin et al., 2014; Zhang et al., 2015) and kaempferol (Lin et al., 2014; Zhang et al., 2015) being the most in vitro active compounds.

Further to genotoxicity, also toxicity endpoints have been evaluated in cell lines treated with Ginkgo biloba extract, including kidney (Hecker et al., 2002), lymphoma (Lin et al., 2014), skin (Hecker et al., 2002; Wang et al., 2015) and stomach (Bai et al., 2015) cells. To the best of our knowledge, only two papers have described the effect of Ginkgo biloba extract in hepatic cell lines (Chao and Chu, 2004; Zhang et al., 2015).

In the present work we investigated cytotoxic and genotoxic effects potentially induced by a well characterized, commercially available, Ginkgo biloba leaf extract (IDN5933 Ginkgoselect®Plus) in human hepatoma HepG2 and in normal liver THLE-2 cell lines. Functional and molecular assays were also performed to investigate the mechanisms of action. Although genotoxic effects of Ginkgo biloba extract have been previously studied in HepG2 cells by Comet assay (Zhang et al., 2015), in light of the variability of the results as a function of the source of the extract, we believed worth testing a further well characterized commercial product using two hepatic cell lines with different sensitivities, as suggested by a multiple cell-line toxicity assessment approach (Shah et al., 2014).

2. Methods

2.1 *Ginkgo biloba extract (IDN5933,Ginkgoselect®Plus) chemical characterization*

The Ginkgo biloba extract (IDN5933, Ginkgoselect® Plus) utilized in this study was produced by INDENA S.p.A. Milan, Italy and contains 24.3% flavone glycosides and 6.1%

of terpene lactones (2.9% bilobalide, 1.38% ginkgolide A, 0.66% ginkgolide B, 1.12% ginkgolide C) as determined by HPLC. Ginkgoselect®Plus is obtained by extracting the dried leaves of Ginkgo biloba using ethanol:water (70:30 v/v). The crude extract is then purified by subsequent concentration to water for the removal of biflavone compounds. Then two consecutive resin columns are used for the elimination of sugars and gingkolic acids respectively.

The obtained product is then dried for residual ethanol and water elimination. Ginkgolic acids are considered unwanted substance of the extract and must be NMT 5 ppm. Only water and ethanol are used as solvents for the preparation of Ginkgoselect® Plus extract.

2.2 *Preparation of Ginkgo extract solution*

A stock solution of Ginkgo extract IDN5933 (Ginkgoselct®Plus,120 mg/mL) was prepared dissolving the powder in dimethyl sulfoxide (DMSO). Working solutions were diluted from stock solution with culture medium to desired concentrations, freshly before use. The final concentration of DMSO in the most concentrated samples did not exceed the 1% v/v as recommended by OECD guidelines (OECD, 2016).

The highest concentration of Ginkgo extract IDN5933 tested in this study (1.2 mg/mL) has been selected on the basis of literature data (Lin et al., 2014) and from our experiments (data not shown) since higher concentrations (2.4 mg/mL and 4.8 mg/mL) highlighted a massive cell death that prevented further analyses.

Extract's concentrations were then progressively reduced to 0.0005 mg/mL because at this concentration, no toxic effect has been detected in the viability assays carried out in both cell lines.

2.3 *Positive control*

In this study, the mycotoxin aflatoxin B1 (AFB1) a known hepatotoxic, mutagenic, genotoxic and carcinogenic substance, was chosen as positive control in all assays, for its ability to reduce cell viability, to induce DNA damage, ROS production and apoptosis in mammalian cells, including HepG2 (El-Golli-Bennour et al., 2010; Liu et al., 2012) as well as to cause changes in gene expression (Shi et al., 2016). AFB1 was prepared similarly to the extract by dissolving the powder in DMSO. Based on concentration-range finding studies, AFB1 concentrations were selected for each test as those inducing a positive but not over response. In particular, the following concentrations have been selected: Viability assay, 10 µM for HepG2 and 100 µM for THLE-2; Colony forming assay, 0.25 µM; Comet assay, 12.5 µM for HepG2 and 25.0 µM for THLE-2; Caspases 3/7 activity, 100µM for both cell lines; Reactive oxygen species assay, 200µM; Array analysis, 100 µM.

2.4 *Cell Cultures*

HepG2 cells, derived from a human hepatocellular carcinoma, and THLE-2, derived from SV40-immortalized normal human liver cells, were purchased respectively from European Collection of Cell Cultures and from American Type Culture Collection respectively. HepG2 cell line is a suitable model for hepatotoxicity studies as it maintains several liver metabolic functions (Nikoloff et al., 2014). THLE-2 cells express phenotypic characteristics of normal adult liver epithelial cells and retain phase I and II enzyme activities, including the ability to metabolize carcinogens to their final carcinogenic metabolites capable of binding DNA (Patel et al., 2015). HepG2 cells were maintained as subconfluent monolayers in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (Hyclone), 2 mM glutamine, 1% non-essential

amino acids and 1% penicillin-streptomycin (10,000 U/mL) from Gibco BRL Life Technologies.

THLE-2 cells were cultured in LHC-8 (Gibco) medium supplemented with 70 ng/mL phosphoethanolamine (Sigma-Aldrich), 5 ng/mL epidermal growth factor (Sigma-Aldrich), 10% fetal bovine serum (Hyclone) and antibiotics as above (Gibco). Both cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.

2.5 *CellTiter-Fluor Cell Viability Assay*

Viability of HepG2 and THLE-2 cells was determined based on quantification of a conserved and constitutive protease activity restricted to intact live cells by CellTiter-Fluor Cell Viability Assay Kit (Promega).

Briefly, HepG2 and THLE-2 cells were seeded and cultured at $1x10^4$ cells/well in black walled 96 well-plates for 24 hrs, and then treated with seven concentrations (0.0005-0.001-0.025-0.05-0.1-0.6-1.2mg/mL) of Ginkgo leaf extract (Ginkgoselect®Plus) for 24, 48 or 72 hrs. In all experiments, non-treated cells (negative control), cells treated with DMSO (1%) (solvent control), cells treated with 10µM or 100µM AFB1 (positive control) were used. Each experimental point was run in triplicate. At the end of treatment, cells were processed according to the manufacturer's instructions. Briefly, CellTiter-Fluor reagent was added to each well after respective treatment time points and plates were incubated 40 min at 37°C before reading fluorescence (Ex 380-400 nm/Em 505 nm) on a Glomax Discover System (Promega).

All experiments were performed in triplicate and the cell viability (%) was expressed as the mean fluorescent intensity of experimental group/the mean fluorescent intensity of solvent control group x 100.

2.6 *Colony Forming Assay*

This in vitro cell viability assay is based on the ability of a single cell to survive and undergo "unlimited" division until forming a colony after treatment with chemicals or physical agents. Viable HepG2 cells were plated at a density of 5×10^2 cells/60 mm Petri plates and allowed to attach for 24 hrs. Then the cells were treated with the Ginkgoselect®Plus extract (0.0005-0.001-0.025-0.05-0.6mg/mL) and incubated for 72 hrs in a humidified CO_2 incubator. After this time, the medium was replaced and cells were grown for further 7 days. Afterwards the cells were fixed in 10% formaldehyde solution (Sigma-Aldrich) and stained with 10% Giemsa Stain modified solution (Sigma-Aldrich). Colonies (>50 cells/colony) were counted under a stereomicroscope and the cytotoxic effect was measured as a decrease in the number of colonies formed after treatment compared to that of solvent control. The results were normalized to the solvent control and expressed in terms of surviving fraction calculated as the percentage of treated cells over solvent control cells. Three independent experiments were performed in triplicate for each concentration and control points.

2.7 *Comet assay*

HepG2 and THLE-2 cells were plated at a density of 5×10^5 cells/60 mm Petri plates and allowed to attach for 24 h. Then DMSO or different concentrations of Ginkgo extract IDN 5933 (0.001-0.025-0.05-0.6 mg/mL) or 12.5μM for HepG2 and 25.0μM for THLE-2 of AFB1 were added to the medium and cell cultures were incubated for 24 h at 37°C in a humidified CO_2 incubator. At the end of treatment, cells were detached using trypsin and resuspended in PBS. The alkaline comet assay was performed essentially as previously described (Cordelli et al., 2016). Two slides were prepared for each experimental point. After lysis, slides were placed in electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH >13), and

left in the solution for 25 min at 4°C. Electrophoresis was carried out at 4°C for 25 min, 27 V (0.8 V/cm) and 300 mA. Slides were then neutralized, fixed and air-dried. Before scoring, slides were stained with 12 µg/mL ethidium bromide (Sigma-Aldrich) and examined at 200X magnification with an Olympus fluorescence microscope. Slides were analyzed blindly with a computerized image analysis system (Delta Sistemi). For each sample, 150 cells were analysed, the percentage of DNA in the tail of the comet (% TI) was used as the parameter for evaluation of DNA damage. Heavily damaged cells i.e. hedgehogs (as determined by visual scoring or cells having more than 80% DNA in the tail), were not included in the measurement but they were counted and their percentage was calculated per sample. Data are presented as the mean ± standard deviation from 3 independent experiments.

2.8 *Caspase 3/7 activity*

Caspase-3 and caspase-7 are both activated during apoptosis and play an important role in the intrinsic and extrinsic pathways of apoptosis in mammalian cells.

Caspase 3/7 activity was assayed in HepG2 and THLE-2 cells to evaluate the activation of apoptotic process after treatment with Ginkgoselect®Plus extract using Caspase-Glo 3/7 luminescent Assay (Promega). The assay was performed following the manufacturer's recommended protocol. Briefly, the HepG2 or THLE-2 cells were seeded at a density of 1×10^4 cells per well in a 96-well white walled plate. Cells were then treated with medium alone, DMSO (solvent control), Ginkgoselect®Plus extract (0.0005-0.001-0.025-0.05-0.1-0.6-1.2 mg/mL) or 100 µM AFB1 (positive control) for 24, 48 or 72 hrs. The luminescence signals were measured with the GloMax-Multi Discover System.

Two independent experiments were performed in triplicate for each concentration and control points.

2.9 *Reactive Oxygen Species*

THLE-2 cells were seeded in white walled 96 well plate at density of 1×10^4/well and treated as described above. For positive control a concentration of 200 µM AFB1 was used. ROS-Glo H_2O_2 assay (Hirst et al., 2015) was performed as directed by the manufacturer (Promega). Luminescence was recorded using a GloMax® Multi Discover System..

Two independent experiments were performed in triplicate for each concentration and control points.

2.10 *RNA extraction and array analysis*

Total RNA was extracted from 1×10^6 THLE-2 cells cultured for 24 hours in the presence of IDN5933 extract (0.025 mg/mL) or AFB1 (100 µM) or solvent DMSO utilizing the mini RNeasy kit (Qiagen). Before further use, RNA was treated with RNAse-free DNAse I (Qiagen). Procedures were carried out essentially as previously described (Pieraccioli et al., 2013). RNA was retro-transcribed into cDNA with RT2 First Strand Kit (Qiagen). cDNA was utilized to carry out a PCR Array (Qiagen,) screening which allows quantitative analysis of the expression of 84 genes whose levels can be altered during hepatic carcinogenesis. The expression of 5 invariant genes, present in the array, was used for normalization. Amplification was carried out in Applied Biosystems thermal cycler 7500 using RT2 SYBR® Green qPCRMastermix (Qiagen) as previously described (Pieraccioli et al., 2016).

Array analysis was repeated twice. Analysis of results was performed using the online accessible Qiagen software (www.SABiosciences.com/pcrarraydataanalysis.php).

2.11 *Quantitative reverse transcriptase PCR analysis*

Total RNA was retro-transcribed using the GoScript Reverse Transcription System kit (Promega) starting from 1µg RNA/sample. cDNA underwent amplification with specific primers for human IGFBP3, CDH1 and CDKN1A (Table 1). Values were normalized for beta-actin expression. Amplification was carried out in Applied Biosystems thermal cycler 7500 using the GoTaqqPCR Master Mix (Promega) essentially as described (Uccini et al., 2005). Results are the mean of 4 independent experiments carried out in triplicate.

2.12 *Statistical analysis*

Statistical comparisons between different groups were carried out using one-way analysis of variance (ANOVA) with Bonferroni correction for post hoc analyses. P values <0.05 after correction were considered statistically significant.

Two-tailed Student's t-test was used for statistical comparisons between negative control and positive control and for PCR array analyses as recommended by the Japanese center for the validation of alternative methods (JaCVAM) (Plappert-Helbig et al., 2015). Differences with p values <0.05 were considered statistically significant. Data are presented as mean value ± standard deviation (SD) of the mean.

3. Results

3.1 *Cell Viability*

The potential effect of the extract from Ginkgo biloba on the viability of HepG2 and THLE-2 cells was evaluated after treatment for 24, 48 and 72 hrs with different concentrations (ranging from 0.0005 to 1.2 mg/mL) of IDN5933 extract.

At these concentrations the extract inhibited cell viability in a dose dependent manner at 24, 48 and 72 hrs. At 24 and 48 hrs of exposure, a significant decrease in the number of viable cells was observed in HepG2 cell line, compared to DMSO control group, after treatment at concentrations of 0.6 and 1.2 mg/mL ($p<0.01$). At 72 hrs of exposure, the extract significantly decreased HepG2 cell viability starting from concentration of 0.05 mg/mL ($p<0.01$ after Bonferroni correction) (Fig. 1 A).

IDN5933 extract significantly affected also cell viability of the normal human liver cell line THLE-2 from 0.05 to 1.2 mg/mL concentrations at all experimental times (Fig. 1 B).

3.2 *Colony Forming Assay*

The surviving of HepG2 cells after treatment with Ginkgoselect® extract was evaluated by the colony forming assay. Figure 2 (Fig. 2) shows the results obtained from three independent experiments.

No significant differences were observed between cultures treated with 0.0005, 0.001 or 0.025 mg/mL extract when compared to the DMSO control. However cell proliferation was significantly (corrected p value <0.01) impaired in a dose dependent manner at the higher concentrations of 0.05 and 0.6 mg/mL compared to control, highlighting a cytotoxic effect at these experimental points.

3.3 *Comet assay*

The sensitivity of HepG2 and THLE-2 cells to genotoxic stress after 24 hrs treatment with extract IDN5933 was evaluated by comet assay. The results obtained applying percentage of DNA in the tail as damage parameter are shown in figure 3. Treatment with concentrations of the extract ranging from 0.001 to 0.6 mg/mL did not display any

significant difference in % tail intensity compared to control cells in both cell lines (Fig. 3 A and B). On the other hand, treatment with AFB1 significantly increased the extent of DNA damage.

3.4 *Caspase 3/7 activity*

In HepG2 and THLE-2 cells treated with Ginkgoselect® extract (0.0005-1.2 mg/mL), there was a dose dependent decrease in caspase 3/7 activity (Fig. 4 A and B) at all considered exposure times (24, 48 and 72 hrs). By contrast, the cells treated with AFB1, that is known to induce apoptosis, exhibited a significant increase in the activation of these enzymes. These findings indicate that the caspase 3/7-dependent apoptotic pathway was not activated in HepG2 and THLE-2 cells after treatment with Ginkgoselect® extract and the decrease in viable cells resulting from the extract treatment was not due to apoptosis.

3.5 *Reactive Oxygen Species (ROS)*

The effect of IDN5933 extract on the production of ROS in THLE-2 cells was investigated (Fig. 5 A). Treatment for 24, 48 and 72 hrs resulted in a dose-dependent increase of luminescence, which reached statistical significance at 0.6-1.2 mg/mL concentrations, indicating pronounced increase of ROS level. At 48 hours of treatment, we detected a significant luminescence increase also at 0.1 mg/mL. Unexpectedly, the ROS increase in treated cells was larger than that in positive control AFB1. To verify the possibility that the generation of ROS might be due to a pro-oxidant reaction of some components of the extract (e.g. polyphenols) with culture medium (Sang et al., 2005; Long et al., 2010), the reactivity of IDN5933 extract with the LCH-8 medium was assayed in a cell-free assay. Such analysis was also carried out with DMEM medium used for HepG2 culture. The

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results of these cell-free analyses highlighted a dose dependent increase in the ROS levels (Fig. 5 B).

3.6 *PCR Array Analysis*

We carried out an array-based transcriptomic analysis of THLE-2 cells treated with Ginkgoselect® extract (0.025 mg/mL for 24 hrs) to detect changes in the expression of 84 liver carcinogenesis-associated genes by reverse transcriptase-quantitative PCR (RT-qPCR). As positive control, we treated THLE-2 cells with AFB1 (100 µM, 24 hrs). Results of these analyses demonstrated that only the expression of IGFBP3 significantly decreased (p≤ 0.05) in cells treated with IDN5933 extract compared to control cells treated with DMSO (Fig. 6A). Treatment with AFB1 affected the expression of a larger number of genes. In fact, the expression of 14 genes (CDH1, CDH13, BCL2L1, FHIT, IGFBP3, IRS1, KDR, MET, PDGFRA, PTK2, RELN, TGFBR2, TLR4, TP53) significantly decreased while that of other 14 (BAX, CASP8, CDKN1A, CXCR4, FAS, FZD7, HRAS, GADD45B, OPCML, RHOA, SFRP2, STAT3, TNFRSF10B, XIAP) increased in AFB1-treated cells compared to control (Fig. 6B). To validate the results of the array experiments, we carried out quantitative reverse transcriptase PCR analyses of the expression of IGFBP3 (which decreased in IDN5933- and in AFB1-treated cells), CDH1 (which decreased in AFB1- and it was invariant in IDN5933-treated cells) and CDKN1A (which increased in AFB1 and it was invariant in IDN5933-treated cells). Results from quantitative reverse transcriptase PCR analyses matched those of the array experiments (Fig. 6C) thus confirming the reliability of the latter. Together these findings suggest that Ginkgo biloba extract causes slight gene expression changes in THLE-2 cells.

4. Discussion

In this study, markers of viability, proliferation, DNA damage, apoptosis and oxidative stress were measured in the hepatocarcinoma HepG2 and normal liver THLE-2 cell lines treated with Ginkgo biloba leaf extract (IDN5933,Ginkgoselect®). Furthermore, analysis of changes in gene expression was carried out in THLE-2 cells. Even if none of the two cell lines possesses the full metabolic capacity of human hepatocytes (Guo et al., 2011), and this may limit the relevance of the study for human risk assessment, nevertheless their use is considered suitable to test liver toxicity (Nikoloff et al., 2014; Patel et al., 2015), since they have advantages over primary cells that have high costs, large variability and limited in vitro proliferation capacity. Moreover, a multiple cell-line toxicity assessment approach (Shah et al., 2014) has been proposed to increase the sensitivity of in vitro testing. In addition THLE-2 cells, which are not tumorigenic when transplanted in vivo, are best suitable to investigate carcinogenicity mode of action.

The in vitro cytotoxicity of the extract was evaluated with two assays that measure cell survival by different endpoints, namely metabolic competence and proliferating capacity. The extract IDN5933 significantly diminished HepG2 and THLE-2 cell viability (Fig. 1) and impaired the in vitro formation of HepG2 colonies (Fig. 2) in a dose-dependent manner. Our results are consistent with those reported by Chao and Chu (Chao and Chu, 2004) who found a dose-dependent decrease of cell proliferation in hepatocarcinoma cell lines HepG2 and Hep3B as well as with those reported by other authors in cells of different origins (Hecker et al., 2002; Lin et al., 2014; Wang et al., 2015; Bai et al., 2015) exposed to different Ginkgo biloba extracts.

To relate these findings with respect to human exposure, first of all it should be noted that, according to ISO 10993-5 (ISO 2009) the in vitro concentrations to be considered frankly

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cytoxic are those inducing over 30 % cell death, as the highest tested concentrations in our experiments (Fig. 1). A comparison between peak plasma level concentrations of representative markers of Ginkgo biloba extract bioavailability, such as bilobalide, ginkgolide A and ginkgolide B in subjects assuming Ginkgo biloba as a dietary supplement (Zadoyan et al., 2012; Woelkart et al., 2010) and those occurring in the culture medium at the toxic concentrations, shows that the latter are between 2 an 4 orders of magnitude higher, suggesting a negligible risk of the extract for cytotoxic effects in humans.

We further evaluated the in vitro genotoxicity of the extract on HepG2 and THLE-2 cells in terms of DNA damage measured by the comet assay. The comet assay is a sensitive method to detect DNA damage in early genotoxicity screening of drug candidates (Witte et al., 2007). Our results showed that the extract Ginkgoselect® failed to induce DNA fragmentation in both HepG2 and THLE-2 cells treated with extract for 24 hrs, even at the highest concentration (0.6 mg/mL) (Fig. 3). Previous studies with neutral comet assay reported significant DNA damage after 4 hrs treatment with Ginkgo extract at concentration of 1 mg/mL in mouse lymphoma L5171Y cells (Lin et al. 2014). Heterogeneous effects of Ginkgo extract on DNA integrity of HepG2 cells have been reported, depending on extract composition. Some extracts induced significant DNA damage at concentrations higher than 0.4 mg/mL while other preparations were ineffective (Zhang et al., 2015). As for most natural products, the quality of biomass, the process of extraction, and other factors can easily result in a different quality and quantity of the constituents of the Ginkgo extract. This may lead to remarkable differences in efficacy as well as in toxicity profiles (Ude et al., 2013). The negative response in terms of DNA damage demonstrated in our study is partially in contrast with other studies (Lin et al., 2014; Zhang et al., 2015). In addition to differences in the cell lines and source of the extract, the different results might depend

upon the lower concentration of flavonoids in Gingkoselect® extract compared to the other studies (24.3% vs 36.9%). Flavonoids, and especially quercetin and kaempferol were, in fact, the components of the extract inducing the highest in vitro genotoxic effects (Lin et al., 2014; Zhang et al., 2015). It should also be stressed that in vivo genotoxicity assays of Ginkgo biloba extract at high doses (up to 2000 mg/kg/day) in mice (Maeda et al., 2014) highlighted a lack of DNA damage in liver cells that is in agreement with our in vitro results.

The induction of apoptosis was evaluated by measuring caspases 3 and 7 activity. Caspases 3 and 7 are central executioners of intrinsic and extrinsic apoptosis in mammalian cells (Chen and Wang, 2002). The treatment with extract IDN5933 showed a significant reduction of caspases 3 and 7 activity in HepG2 and THLE-2 cells at all concentrations and time points, possibly due to non-apoptosis-dependent reduction of viable cells evidenced by the viability assay. On the contrary, treatment with AFB1 induced a significant increase of activity of these enzymes compared to solvent control. The lack of pro-apoptotic effect is further confirmed by the array analysis in which we could not detect any significant change in the expression of the apoptosis-associated genes present in the array. It should be noticed that the same analysis highlighted significant changes in the expression of apoptosis-associated genes, among which BAX, CASP8 and FAS, in cells treated with AFB1. These results emphasize the importance of the type of cells selected for analysis. In fact, an increase in apoptosis has been reported in various non-hepatic cells treated with Ginkgo biloba extracts (Wang et al., 2015; Bai et al., 2015; Kim et al., 2005).

In the present study, treatment of THLE-2 cells with the extract IDN5933 resulted in a pronounced dose-dependent production of ROS at all experimental times, much more evident than that induced by positive control AFB1. These results are presumably dependent on a chemical oxidative reaction between the Ginkgo biloba extract (most likely

of its polyphenolic component) and the culture medium (Sang et al., 2005; Long et al., 2010; Babich et al., 2009). The ability of the Ginkgo extract IDN5933 in inducing ROS in a cell-free manner has been confirmed by exposing LCH-8 and DMEM culture media to the same range of extract concentrations utilized in viability experiments for 24 hours (Figure 5). It should be stressed that no increase in ROS level was detected in cell free experiments in which culture media were exposed to AFB1 or DMSO. Thus, the observed increase in ROS levels in in vitro cultures treated with Ginkgo biloba extract is unlikely to be relevant for the use of this natural product as dietary supplement.

The extracellular production of ROS could explain the reduction in cell viability that we detected at high doses of the extract. In fact, a mild exposure to the extract causes a limited ROS production, so that the cells are able to deal with this stress by activating antioxidant defense systems. On the contrary, higher extract concentrations overwhelm these systems, causing oxidative damage leading to cell death.

Array-based transcriptomic analysis of THLE-2 primary hepatocytes underscored limited changes in the expression pattern of genes involved in hepatic tumorigenesis. Indeed, only the expression of IGFBP3 was found significantly down-regulated compared to controls. IGFBP3 is a gene that encodes for a protein that binds to and modulate the activity of IGF1 and IGF2 (Ranke, 2015). In liver cancer, the expression of IGFBP3 decreases compared to normal hepatic tissue (Subramaniam et al., 2010). Nevertheless, it is unlikely that the slight variation in IGFBP3 level detected in IDN5933-treated cells may cause functional effects since IGFBP3 is the only gene in the panel whose expression changed. On the contrary, significant up- and down-modulation in the expression of 28 genes were detected in cells treated with the known hepatocarcinogen AFB1. The sensitivity of the array analysis was confirmed by the results obtained in THLE-2 cells treated with AFB1. We detected a

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significant variation in the level of expression of 28 genes (14 up-regulations and 14 down regulations). Modulated genes belong to pathways such as Apoptosis, EGFR signaling, MET/HGF signaling, RAS/RAF/MAP kinase signaling, EGFR signaling and G-protein signaling which are involved in the onset and in the progression of hepatocellular carcinoma (HCC), as well as of other forms of hepatocarcinogenesis.

Our results in human hepatic cells do not show effects of Ginkgo biloba on DNA damage or carcinogenesis-related gene expression. These findings favor the hypothesis of a CAR-mediated mechanism underlying the carcinogenic effect of Ginkgo biloba extract in rodents, put forward by Maeda and co-workers (Maeda et al., 2014, 2015), over alternative pathways, such as those triggered by DNA damage or oxidative stress. Since a CAR-mediated mechanism is not deemed relevant for carcinogenicity in humans (LeBaron et al., 2014), our results are reassuring regarding possible human risks, at least for the specific extract tested. In addition, although the results presented herein show some cytotoxic effects, the concentrations required to induce these effects are 2 to 4 order of magnitude higher than those reached in plasma of subjects assuming Ginkgo biloba as a dietary supplement. Importantly, since the available data underline the heterogeneity in genotoxic and cytotoxic effects brought about by different Ginkgo extract preparations, a rigorous control in its quality and chemical composition seems necessary for its nutraceutical use in humans.

Acknowledgements

Conflict of interest

The study was financially supported by Indena S.p.A., the company that produces the Ginkgo extract IDN5933, made available to ENEA laboratories for testing.

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Figure captions

Fig. 1. Effect of Ginkgo biloba leaf extract (IDN5933, Ginkgoselect®) on viability of HepG2 (A) and THLE-2 (B) cells, after treatment with different concentrations (range: 0.0005-1.2 mg/mL) for 24, 48 and 72hrs. Data are presented as the mean percent of DMSO control ± Standard Deviation (SD) of three independent experiments, carried out in triplicate. Significant differences between each extract concentration and the control (DMSO) were evaluated using the one-way analysis of variance Anova followed by Bonferroni post hoc test (*$p < 0.05$ and **$p < 0.01$ after Bonferroni correction). Differences between positive control (10 µM and 100 µM AFB1 for HepG2 and THLE-2, respectively) and the solvent control (DMSO) were statistically evaluated by two tail Student's t-test (**$p<0.0001$).

Fig. 2. Colony Forming Assay results after treatment of HepG2 cells with Ginkgo biloba leaf extract (IDN5933, Ginkgoselect®) for 72 hrs. Surviving fraction was expressed as a percentage of the control and histograms were obtained as mean ± Standard Deviation (SD) of three independent experiments done in three replicates for each treatment. Significant differences between each extract concentration and the control (DMSO) were evaluated using the one-way analysis of variance Anova followed by Bonferroni post hoc test (**$p < 0.01$). Differences between positive control (AFB1) and the solvent control (DMSO) were statistically evaluated by two tail Student's t-test (**$p<0.0001$).

Fig. 3. Comet Assay results after treatment of HepG2 (A) and THLE-2 (B) cells with Ginkgo biloba leaf extract (IDN5933, Ginkgoselect®) for 24 hrs. Columns and bars represent the mean percentage ± Standard Deviation (SD) of DNA in the tail of the comet

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(%TI) from 3 independent experiments. Aflatoxin B1 (AFB1) 12.5 µM and 25.0 µM respectively in HepG2 and THLE-2 cells, was used as a positive control. Significant differences between each extract concentration and the control (DMSO) were evaluated using the one-way analysis of variance Anova followed by Bonferroni post hoc test. Differences between positive control (AFB1) and the solvent control (DMSO) were statistically evaluated by two tail Student's t-test (**$p<0.0001$, * $p<0.05$).

Fig. 4. Effect of Ginkgo biloba leaf extract (IDN5933, Ginkgoselect®) on caspase 3/7 activation of HepG2 (A) and THLE-2 (B) cells after treatment with different concentrations (range: 0.0005-1.2 mg/mL) for 24, 48 and 72 hrs. Caspase 3/7 activity level was expressed as a percentage of DMSO control and data were expressed as mean ± Standard Deviation (SD) of two independent experiments, carried out in triplicate. Significant differences between each extract concentration and the control (DMSO) were evaluated using the one-way analysis of variance Anova followed by Bonferroni post hoc test (*$p <0.05$ and **$p < 0.01$). Differences between positive control (AFB1) and the solvent control (DMSO) were statistically evaluated by two tail Student's t-test (**$p<0.0001$).

Fig. 5. A: ROS generation after treatment of THLE-2 cells with Ginkgo biloba leaf extract (IDN5933, Ginkgoselect®) at different concentrations (range: 0.0005-1.2 mg/mL) after 24, 48 and 72hrs incubation. Results are expressed as a percentage of DMSO control and represent mean ± Standard Deviation (SD) of two independent experiments, carried out in triplicate. Significant differences between each extract concentration and the control (DMSO) were evaluated using the one-way analysis of variance Anova followed by Bonferroni post hoc test (*$p < 0.05$ and **$p < 0.01$).

Significant differences between positive control (AFB1) and the solvent control (DMSO) were statistically evaluated by two tail Student's t-test (*p<0.05 and **p<0.0001). **B: ROS generation in cell-free assay by Ginkgo biloba leaf extract (IDN5933, Ginkgoselect®) at different concentrations (range: 0.0005-1.2 mg/mL) after 24 hrs incubation in LCH-8 medium (left) and DMEM medium (right).** Results are expressed as a percentage of DMSO control and represent mean ± Standard Deviation of one experiment carried out in triplicate.

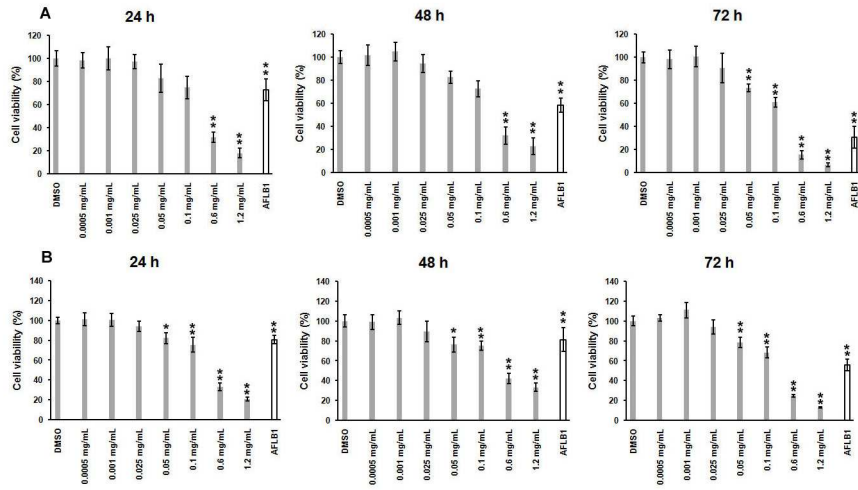**Fig. 6. PCR array results from THLE-2 cells treated with 0.025 mg/mL of Ginkgo biloba leaf extract (IDN5933, Ginkgoselect®)(A) and 100 μM AFB1 (B) for 24 hours.** Values are the mean of two independent experiments ± standard deviation. p values (two tailed Student's t test) are reported only for those genes whose variation reached statistical significance (< 0.05). n.s. = not significant. Dark grey and light grey background indicate an increase and a decrease respectively in the level of expression of DMSO-treated control taken as 1. C: quantitative RT-PCR analysis of IGFBP3, CDH1 (E-cadherin) and CDKN1A (p21) expression in THLE-2 cells treated with solvent DMSO, extract IDN5939 and AFB1 at the same concentrations and for the same time as in A and B. Values were normalized respect to the expression of the beta-actin gene. Asterisks indicate a statistically significant difference (two tailed Student's t test, p<0.01). Values are the mean ± standard deviation of 4 independent experiments carried out in triplicate.

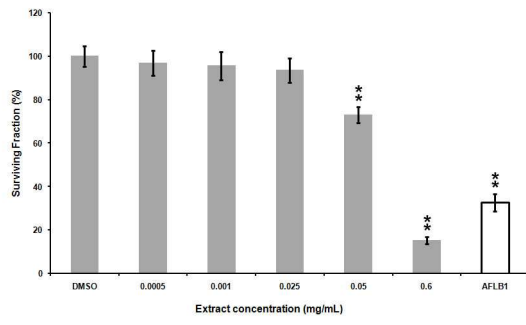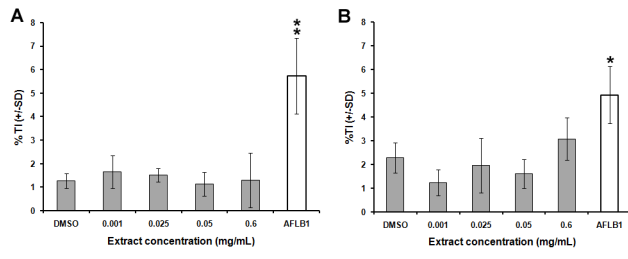## Table 1. Primers utilized for qPCR

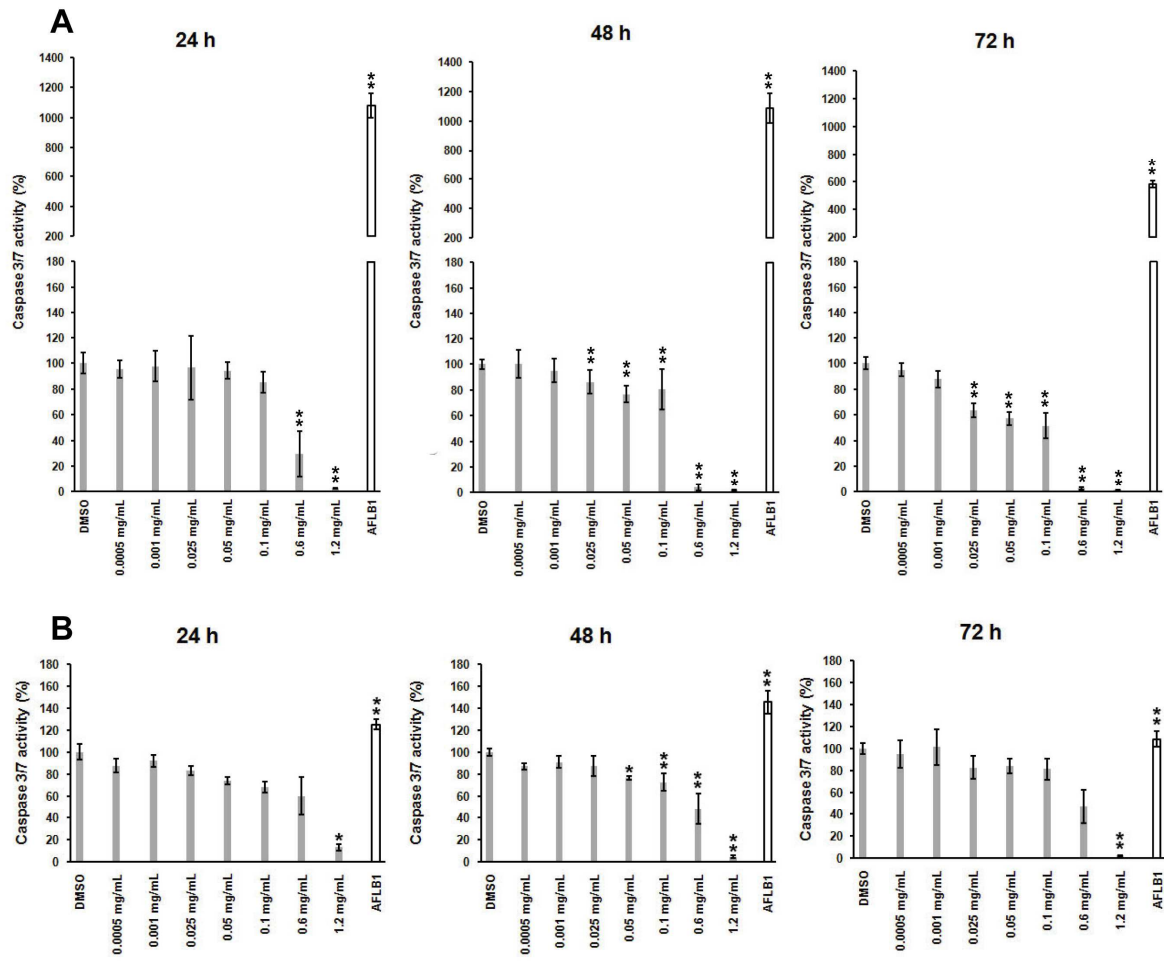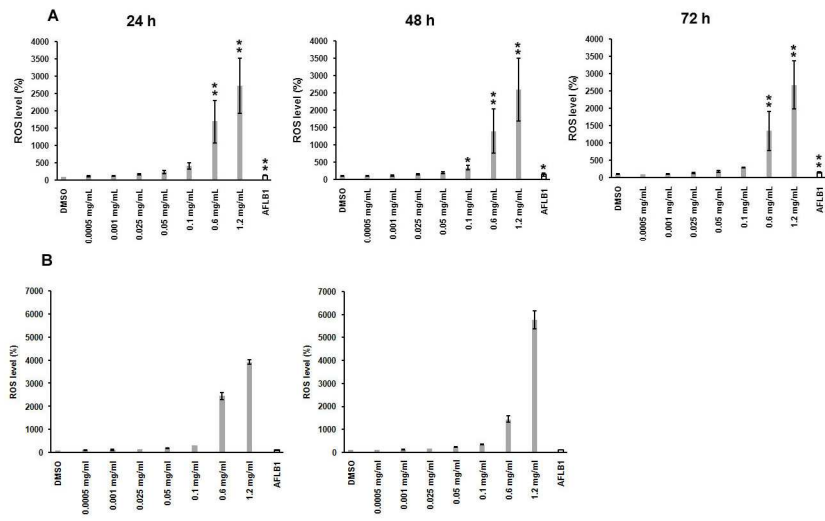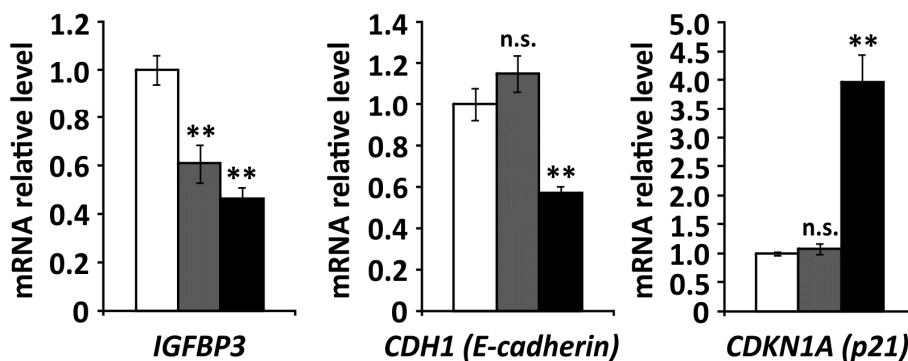| Name	Sequence (from 5' to 3')
human beta-actin F	GTTGCTATCCAGGCTGTGCTA
human beta-actin R	AATGTCACGCACGATTTCCCG
human IGFBP3 F	CTCTGCGTCAACGCTAGTGC
human IGFBP3 R	CGGTCTTCCTCCGACTCACT
human CDH1 F	CCGCTGGCGTCTGTAGGAAGG
human CDH1 R	GGCTCTTTGACCACCGCTCTCC
human CDKN1A F	TGAGCGATGGAACTTCGAC
human CDKN1A R	ACAAGACAGTGACAGGTCC

F= forward; R= reverse

A

Ginkgo biloba

ADAM17	AKT1	ANGPT2	BAX	BCL2	BCL2L1	BID	BIRC2	BIRC5	CASP8	CCL5
0,96 ± 0,09	0,9 ± 0,01	0,76 ± 0,16	1,07 ± 0,03	1,27 ± 0,19	0,93 ± 0,07	0,96 ± 0,10	0,85 ± 0,1	1,08 ± 0,11	0,98 0,05	1,15 ± 0,11
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CCND2	CDH1	CDH13	CDKN1A	CDKN1B	CDKN2A	CFLAR	CTNNB1	CXCR4	DAB2IP	DLC1
1,19 ± 0,15	1,17 ± 0,1	1,00 ± 0,05	1,18 ± 0,10	0,96 ± 0,00	0,98 ± 0,08	0,99 ± 0,03	1,04 ± 0,02	1,31 ± 0,06	0,94 ± 0,04	0,97 ± 0,02
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
EGF	EGFR	EP300	FADD	FAS	FHIT	FLT1	FZD7	GADD45B	GSTP1	HGF
2,14 ± 0,78	0,86 ± 0,02	0,90 ± 0,12	1,04 ± 0,02	0,96 ± 0,05	1,23 ± 0,06	1,17 ± 0,18	0,99 ± 0,01	0,97 ± 0,06	1,21 ± 0,14	1,06 ± 0,07
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
HRAS	IGF2	IGFBP1	IGFBP3	IRS1	ITGB1	KDR	LEF1	MCL1	MET	MSH2
1,10 ± 0,05	0,83 ± 0,05	1,09 ± 0,01	**0,63 ± 0,01**	0,75 ± 0,20	1,01 ± 0,09	0,97 ± 0,01	0,71 ± 0,47	1,06 ± 0,13	0,97 ± 0,03	1,02 ± 0,21
n.s.	n.s.	n.s.	≤0,05	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
MTDH	MYC	NFKB1	NRAS	OPCML	PDGFRA	PIN1	PTEN	PTGS2	PTK2	PYCARD
0,97 ± 0,09	1,08 ± 0,17	1,02 ± 0,06	1,11 ± 0,04	1,44 ± 0,54	0,98 ± 0,04	0,98 ± 0,06	1,03 ± 0,17	1,08 ± 0,07	1,09 ± 0,03	1,05 ± 0,06
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
RASSF1	RB1	RELN	RHOA	RUNX3	SFRP2	SMAD4	SMAD7	SOCS1	SOCS3	STAT3
0,84 ± 0,14	0,99 ± 0,11	1,11 ± 0,01	1,18 ± 0,08	1,06 ±0,07	0,97 ± 0,23	1,07 ± 0,10	0,79 ±0,04	1,05 ± 0,09	0,83 ± 0,16	1,04 ± 0,07
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
TERT	TGFA	TGFB1	TGFBR2	TLR4	TNFRSF10B	TNFSF10	TP53	VEGFA	WT1	XIAP
1,06 ± 0,07	0,93 ± 0,06	1,08 ± 0,03	0,95 ± 0,01	0,96 ± 0,05	0,96 ± 0,01	0,87 ± 0,07	1,10 ± 0,04	0,99 ± 0,14	0,74 ± 0,04	0,97 ± 0,04
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

B

Aflatoxin B1

ADAM17	AKT1	ANGPT2	BAX	BCL2	BCL2L1	BID	BIRC2	BIRC5	CASP8	CCL5
1,20 ± 0,05	0,89 ± 0,05	1,58 ± 1,15	**1,39 ± 0,06**	0,89 ± 0,20	**0,68 ± 0,04**	1,52 ± 0,22	1,58 ± 0,22	1,03 ± 0,15	**1,18 ± 0,03**	1,14 ± 0,21
n.s.	n.s.	n.s.	≤0,05	n.s.	≤0,05	n.s.	n.s.	n.s.	≤0,05	n.s.
CCND2	CDH1	CDH13	CDKN1A	CDKN1B	CDKN2A	CFLAR	CTNNB1	CXCR4	DAB2IP	DLC1
0,70 ± 0,12	**0,48 ± 0,01**	**0,45 ± 0,02**	**3,19 ± 0,54**	1,34 ± 0,21	0,99 ± 0,13	1,00 ± 0,13	1,40 ± 0,11	**2,46 ± 0,25**	1,00 ± 0,07	0,91 ± 0,04
n.s.	≤0,01	≤0,01	≤0,05	n.s.	n.s.	n.s.	n.s.	≤0,05	n.s.	n.s.
EGF	EGFR	EP300	FADD	FAS	FHIT	FLT1	FZD7	GADD45B	GSTP1	HGF
0,80 ± 0,11	0,90 ± 0,14	1,17 ± 0,25	1,14 ± 0,12	**2,02 ± 0,23**	**0,13 ± 0,02**	1,55 ± 0,59	**1,41 ± 0,01**	**1,81 ± 0,20**	1,39 ± 0,19	0,91 ± 0,05
n.s.	n.s.	n.s.	n.s.	≤0,05	≤0,01	n.s.	≤0,01	≤0,05	n.s.	n.s.
HRAS	IGF2	IGFBP1	IGFBP3	IRS1	ITGB1	KDR	LEF1	MCL1	MET	MSH2
1,86 ± 0,17	1,03 ± 0,04	0,99 ± 0,21	**0,33 ± 0,07**	**0,57 ± 1,03**	1,08 ± 0,08	**0,53 ± 0,01**	0,73 ± 0,048	1,69 ± 0,26	**0,76 ± 0,03**	1,29 ± 0,12
≤0,05	n.s.	n.s.	≤0,01	≤0,01	n.s.	≤0,01	n.s.	n.s.	≤0,01	n.s.
MTDH	MYC	NFKB1	NRAS	OPCML	PDGFRA	PIN1	PTEN	PTGS2	PTK2	PYCARD
1,22 ± 0,16	0,69 ± 0,11	1,27 ± 0,09	1,17 ± 0,10	**7,11 ± 1,49**	**0,58 ± 0,01**	1,23 ± 0,12	1,10 ± 0,11	1,74 ± 0,29	**0,69 ± 0,03**	1,16 ± 0,10
n.s.	n.s.	n.s.	n.s.	≤0,05	≤0,01	n.s.	n.s.	n.s.	≤0,01	n.s.
RASSF1	RB1	RELN	RHOA	RUNX3	SFRP2	SMAD4	SMAD7	SOCS1	SOCS3	STAT3
1,00 ± 0,13	1,43 ± 0,23	**0,34 ± 0,05**	**1,17 ± 0,04**	0,91 ± 0,05	**1,66 ± 0,16**	1,22 ± 0,16	0,96 ± 0,04	1,51 ± 0,45	1,11 ± 0,06	**1,38 ± 0,06**
n.s.	n.s.	≤0,01	≤0,05	n.s.	≤0,05	n.s.	n.s.	n.s.	n.s.	≤0,05
TERT	TGFA	TGFB1	TGFBR2	TLR4	TNFRSF10B	TNFSF10	TP53	VEGFA	WT1	XIAP
0,91 ± 0,05	1,01 ± 0,03	0,92 ± 0,08	**0,46 ± 0,01**	**0,71 ± 0,01**	**1,56 ± 0,01**	0,72 ± 0,10	**0,80 ± 0,02**	1,06 ± 0,17	0,91 ± 0,44	**1,41 ± 0,00**
n.s.	n.s.	n.s.	≤0,01	≤0,01	≤0,01	n.s.	≤0,01	n.s.	n.s.	≤0,01

C

□ DMSO ▨ Ginkgo biloba ■ Aflatoxin B1

Highlights

- Multiparameter analyses of Ginkgo biloba extract IDN5933 have been performed

- The extract tested does not show genotoxic and carcinogenesis-inducing effects

- For nutraceutical use, the composition of extracts should be strictly controlled