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Significant decrease in the viability and tumor stem cell marker expression in tumor cell lines treated with curcumin

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1. Introduction

Approximately 14.1 million new cancer cases and 8.2 million cancer-related deaths worldwide were estimated by GLOBOCAN 2012 (Ferlay et al., 2015). It has been predicted that the incidence of new cancer cases will substantially increase to 20 million by 2025 (Cancer, 2013).

Prevention, early diagnosis and effective treatment are fundamental for cancer management. The limitations of current treatment methods to eliminate cancer cells, highlight the importance of new treatment strategies. A series of substances derived from vegetables, grains and fruits has been shown to prevent or treat most of cancer types by effectively targeting cancer cells (Bhanot et al., 2011; Buhrmann et al., 2015, 2016; Busch et al., 2012; Shakibaei et al., 2012). Curcumin (molecular weight of 368.38 g/mol corresponding to a molecular formula of $C_{21}H_{20}O_6$) is the major constituent derived from the rhizomes of turmeric (Anand et al., 2008). Commercial curcumin has three major phenolic analogues such as curcumin (diferuloylmethane, curcumin I) (82 %), desmetoxycurcumin (p-hydroxycinnamoylferuloyl-methane, curcumin II) (15–17 %) and bisdemetoxycurcumin (di-p-

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hydroxycinnamoylmethane, curcumin III) (3–5 %) together referred to as curcuminoids (Anand et al., 2008). Furthermore, several tautomers including keto and enol forms have been distinguished for curcumin which are influenced by pH and polarity changes in solution or solid state (Kawano et al., 2013). Many scientific studies have supported notable properties of curcumin including anti-microbial, anti-carcinogenic, anti-inflammatory and anti-oxidative activities (Prasad et al., 2014a; Shakibaei et al., 2014, 2007; Shakibaei et al., 2015). Curcumin has been well-established as an anticancer agent by a wide range of laboratorial and clinical experiments, for instance, Shakibaei et al. (2015) showed that curcumin potentiated the antitumor activity of 5-fluorouracil against a colorectal cancer cell line *in vitro* (Shakibaei et al., 2015).

Cancer stem cells (CSCs) with the ability of self-renewal, multi-potential of differentiation and other stem cell properties are regarded as emerging therapeutic targets (Chen et al., 2013; Subramaniam et al., 2010). It has been discovered that cancer stem cells, as a small subpopulation of cancer cells, play prominent roles in the initiation and progression of cancer, angiogenesis, invasion, metastasis, resistance to therapy and recurrence of cancer (Gerger et al., 2011; Klarmann et al., 2009; Malik and Nie, 2011; Sampieri and Fodde, 2012; Tysnes and Bjerkvig, 2007; Zhao et al., 2011). A plethora of studies published in the last decade, have supported the potential of curcumin as well as its modified forms to target CSCs in several types of cancer cell cultures alone or in combination with other anticancer agents (Buhrmann et al., 2014; Li and Zhang, 2014; Shakibaei et al., 2014). The effect of curcumin on CSCs may be related to its ability to directly or indirectly affect self-renewal pathways, tumorsphere formation, tumor microenvironment, enzyme activities and cell-surface markers (Buhrmann et al., 2014; Li and Zhang, 2014; Shakibaei et al., 2014).

Recently, various cancer stem cell biomarkers *e.g.* CD44, CD133, ALDH1 have been extensively studied among several types of cancer (Buhrmann et al., 2014; Klonisch et al., 2008; Shakibaei et al., 2014). The CD44 glycoprotein, expressed in multiple isoforms, is involved in many cell signaling pathways related to all stages of cancer (Buhrmann et al., 2014; Williams et al., 2013). Accordingly, CD44 has been proposed as a parameter for cancer prevention, detection, prognosis and screening cancer stem cells response to a variety of treatment modalities (Blacking, 2013; Negi et al., 2012). Expression of glycoprotein CD133 is associated with maintaining stem cell-like properties in cancer cells. Indeed, its expression on the cancer cells was reported as an important marker for prognosis and prediction of treatment outcome (Grosse-Gehling et al., 2013; Glumac and LeBeau, 2018). The enzyme Aldehyde dehydrogenase 1 (ALDH1) may protect the cells from oxidative insults and through converting retinol to retinoic acid is involved in regulating cell proliferation (Huang et al., 2009). ALDH1 was reported as a potential biomarker for human colon cancer and is used as a prognostic marker (Chen et al., 2011; Tomita et al., 2016).

The use of curcumin as a therapeutic agent is limited by its bioavailability and bioefficacy which have been subject to a large number of research projects. All the evidence available to date from *in vitro* and *in vivo* studies indicates that the particular concerns are the poor stability and low bioavailability of curcumin (Anand et al., 2007). However, better knowledge of the stability of curcumin in cell culture media or body compartments (e. g. blood, tissues organs) is an important prerequisite for novel therapy development as there is a strong relationship between the concentration of curcumin and its ability to affect the biological systems. Indeed, several strategies, for example, utility of adjuvants, liposomes, phospholipid complexes, nano-particles or structural analogues of curcumin have been developed in order to overcome the above-mentioned problems (Prasad et al., 2014b).

At the present study, the time- and dose- dependent effect of curcumin on induction of cell death and expression of cancer stem cell markers CD44, CD133 and ALDH1 was investigated *in vitro*. Additionally, the stability of curcumin and curcuminoids was examined in different culture systems.

2. Materials and methods

2.1. Reagents and antibodies

RPMI-1640 and DMEM (4.5 g/L glucose) were obtained from GIBCO (NY, USA) and then supplemented with 10 % (v/v) heat inactivated fetal calf serum (FCS; GIBCO, NY, USA), 50 mg/ml streptomycin (Sigma, MO, USA), 50 U/ml penicillin (Sigma, MO, USA), 2.5 mg/ml amphotericin B and 2 mM L-glutamine (Sigma, MO, USA). Curcumin with a purity greater than 95 % was purchased from Indsaff (Punjab, India). Curcumin was dissolved in dimethylsulfoxide (DMSO; Sigma, MO, USA) at a stock concentration of 5000 μM and stored at −20 °C until used. This stock was then diluted to different concentrations (1, 5, 10, 20, 40 and 80 μM) in supplemented growth medium for the experiments. Mouse anti-human CD44-FITC antibody and mouse IgG2b/FITC were obtained from Becton Dickinson (BD; San Diego, CA). Monoclonal CD44, CD133 and ALDH1 for immunofluorescence and western blotting were obtained from antibodies online (Munich, Germany). All antibodies were used at concentrations and dilutions recommended by the manufacturer.

2.2. Cell lines and cell culture

Human lung adenocarcinoma cell line (A549), human colon carcinoma cell line (HCT116) and human chronic lymphocytic leukemia (183E95) were purchased from Pastor Institute (Tehran, Iran). The adherent cells, A549 and HCT116, were cultured as monolayers in 25 cm^2 tissue culture flasks in supplemented DMEM and RPMI-1640, respectively. Suspension culture of 183E95 performed in 25 cm^2 tissue culture flasks in supplemented RPMI-1640. The flasks were maintained at 37 °C in a 95 % humidified atmosphere containing 5 % (v/v) CO_2. The mediums were changed every three days and 70–80 % confluent adherent cells were passaged using 0.05 % trypsin/EDTA (Sigma, MO, USA) (Langdon, 2010).

2.3. Cell viability assay

The viability of three cell lines in response to curcumin treatment was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, MO, USA) method (Csaki et al., 2008). Briefly, Cells were seeded into 96-well tissue culture plates at a density of 1000 per well and treated the following day with different concentrations of curcumin (1, 5, 10, 20, 40 and 80 μM) in triplicate for 12 and 24 h. Control cells were treated with DMSO to a final concentration of 0.8 % (v/v). Subsequently, MTT solution (5 mg/ml) was added to each well in an amount equal to 10 % of the culture volume and the reaction was terminated by incubation for 4 h at 37 °C. The formazan complex was solubilized by adding MTT solvent to each well and shaken for 15 min at room temperature. The absorbance of colored solution in each well was measured at a wavelength of 570 nm by an ELISA reader (Awareness Technology; INC, USA). The results were presented as percentage survival compared to the corresponding controls.

2.4. Giemsa staining assay

Adherent cells were seeded into MBST tissue culture plates at a density of 2 × 106 and incubated overnight to allow them to adhere as monolayers before being treated with different concentrations of curcumin (1, 5, 10, 20, 40 and 80 μM). Each culture plate has deepened sites for 12 glass circular discs with 1.2 cm diameter. After treatment for 12 and 24 h, the cells grown on the discs were first fixed with methanol (Merck, Darmstadt, Germany) and then stained with Giemsa (Sigma, MO, USA). The treated- suspension cells, after harvesting into 15 mL centrifuge tube, spun at 1000 RPM (200 *g*) for 5 min. Next, the supernatant was aspirated and the cells were resuspended in 0.5 mL of

FCS and 1 drop of the suspension was placed on the mentioned discs for fixation and Giemsa staining processes. Control cells were processed in parallel in the absence of curcumin and were treated with DMSO to a final concentration of 0.8 % (v/v).The analysis was performed under light microscope connected to a video camera for capturing images (Su et al., 2010).

2.5. Flowcytometry assay for CD44 expression

Lung, colon and leukemia cancer cell lines were subjected to direct immunofluorescence staining by a FACS Calibur machine (BD, San Diego, CA) according to the standard protocol (Roudi et al., 2014). Briefly, cells were seeded into 6-well tissue culture plates at a density of 5×10^5 per well and cultured until 50 % confluence was reached. Subsequently, the cells were treated with different concentrations of curcumin (1, 5, 10, 20, 40 and 80 μM) for 12 and 24 h. Control cells were treated with DMSO to a final concentration of 0.8 % (v/v). Control and treated cells were washed and then resuspended in PBS followed by trypsinization (only adhered cells). To characterize the CD44 expressed on cancer stem cells, mouse anti-human CD44-FITC antibody against common epitope to all CD44 isoforms was applied according to the manufacturer's instruction. Mouse IgG2b/FITC was used as an isotype control. After 30 min incubation in the dark at room temperature, the cell samples were fixed using ice cold 70 % ethanol (Merck, Darmstadt, Germany) for 60 min, washed twice with PBS and then resuspended with propidium iodide (10 mg/ml) and ribonuclease A (0.1 %) in PBS for 30 min. Finally, the expression of CD44 was evaluated with a cell count of 20,000 cells per sample.

2.6. HPLC assay

The HPLC analysis was carried out with Waters 2695 HPLC system (Waters, Milford, USA) equipped with C18 column (4.6 × 250 mm, 5 μm particle size) (Waters, Milford, USA) and photodiode array detector (PDA; Waters, Milford, USA) at 430 nm. The mobile phase was composed of acetonitrile/water/acetic acid (60:39:1 v/v/v). The system was run at a constant flow rate of 1 ml/min and the injection volume was 20 μl. The calibration samples at concentration of 5, 10, 20, 40 and 80 μM were provided by the injection of each of the working solutions and the linearity was obtained in the range of 5–80 μM of curcumin ($r^2 = 0.97$). In order to determin the curcuminoid concentrations, four groups were considered in 12-well tissue culture plates as follows: RPMI-1640 (1 mL); RPMI-1640 (1 mL) + A549 cells (3×10^5 cells/well); RPMI-1640 (1 mL) + 10 % FCS; RPMI-1640 (1 mL) + 10 % FCS + A549 cells (3×10^5 cells/well). Sampling was performed at three hour intervals during a period of 24 h after adding an initial concentration of 80 μM curcumin into each well. By triple-injection of each collected sample into the HPLC machine, curcuminoid concentrations were measured based on a linear calibration curve. The reason to choose A549 cells for the HPLC technique was their high resistance to curcumin compared to the other investigated cell lines in the present study (Jayaprakasha et al., 2002).

2.7. Immunofluorescence

The expression pattern of CD44, CD133 and ALDH1 in HCT116 monolayer culture was investigated with Immunofluorescence technique as described by Buhrmann et al. (2014). Briefly, colon cancer cells were seeded at a density of 4×10^4 cells/ml on glass plates and grown to 60–70 % confluence. For the experiment, cells were either left untreated or treated with curcumin (5μM) for 60 min. For immunofluorescent staining, cells were fixed with methanol for 10 min, followed by incubation with 0.5 % Triton X-100/PBS (3 min.) for cell membrane permeabilization and with 1 % BSA/PBS for unspecific blocking (10 min.). Samples were incubated overnight with primary antibodies (dilution of 1:80 in 1 %BSA/PBS) in a humid chamber at 4 °C, washed three times with PBS and incubated with fluorescent labelled secondary antibodies (1:100 dilution) for 1.5 h. Finally, nuclear staining was performed with DAPI, samples mounted with Fluoromount mountant and visualised under a fluorescent microscope (Leica, Wetzlar, Germany).

2.8. Western blot analysis

To investigate the effect of curcumin on expression of colon cancer stem cell markers (CD44, CD133 and ALDH1) in HCT116 western blot investigation was carried out as described by Shakibaei et al. (2014). HCT116 cells in monolayer culture (2×10^5 cells/ml) were left untreated or treated with curcumin (5μM) for 5, 10, 30 and 60 min. and total proteins were extracted with lysis buffer (50 mM Tris/HCl, pH 7.2; 150 mM NaCl; 1 % (v/v) Triton X-100; 1 mM Na3VO4; 50 mM Na4P2O7; 100 mM NaF; 0.01 % (v/v) aprotinin; 4 μg/ml pepstatin A; 10 μg/ml leupeptin; 1 mM PMSF). Proteins were separated by SDS-PAGE-electrophoreses (7.5 % polyacrylamide gels) under reducing conditions, transferred onto nitrocellulose membrane with a transblot apparatus (Bio-Rad, Munich, Germany) and incubated with primary antibodies overnight (1:10 000), followed by incubation with secondary antibodies for 1.5 h. Finally, nitrocellulose membranes were washed with 0,1 M Tris (PH: 9.5; containing 0.05 M MgCl2 and 0.1 M NaCl) three times and antibody-antigen complexes visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (p-toluidine salt; Pierce, Rockford, IL, USA).

2.9. Statistical analysis

Each experiment was performed three times independently in order to certify repeatability and reliability of the results. The numerical results are expressed as mean values ± standard deviations (SD) derived from three independent experiments. Data were analyzed by Student's t-test, one-way ANOVA or repeated-measures two-way ANOVA. A threshold P-value less than 0.05 was assumed to be statistically significant.

3. Results

3.1. Curcumin suppressed viability and proliferation of cancer cells

In order to assess the effect of curcumin on cell viability and proliferation, HCT116, A549 and 183E95 cells were exposed to different doses of curcumin (1, 5, 10, 20, 40 and 80 μM) for 12 and 24 h. Viability and proliferation of the cells was analyzed by MTT assay. As shown in Fig. 1, the results demonstrated clearly that the exposure to curcumin suppressed cell viability and proliferation in a time- and dose-dependent manner in all three cell lines. The IC50 values are means ± SD of three individual determinations each performed in triplicate. The IC50 values at 12 h exposure of curcumin were approximately 40.33 ± 5.69 μM for A549, 18.32 ± 0.84 μM for HCT116 and 10.54 ± 0.38 μM for 183E95 cells. At 24 h exposure of curcumin, the IC50 values were 29.46 ± 4.59 μM for A549, 15.85 ± 0.79 μM for HCT116 and 9.25 ± 0.32 μM for 183E95 cells. At both 12 and 24 h of curcumin treatment, the greatest and the least suppression in viability and cell proliferation was observed in 183E95 and A549 cells, respectively. In addition, the authors evaluated the morphological changes of the cells treated with curcumin by Giemsa staining. Fig. 2 showed the morphological change in form of rounding and growth inhibition in example HCT116 cell line. A positive correlation was also observed between different doses of curcumin (1, 5, 10, 20, 40 and 80 μM) and cytotoxicity patterns in each of the tested cell lines in both 12 and 24 h treatment periods.

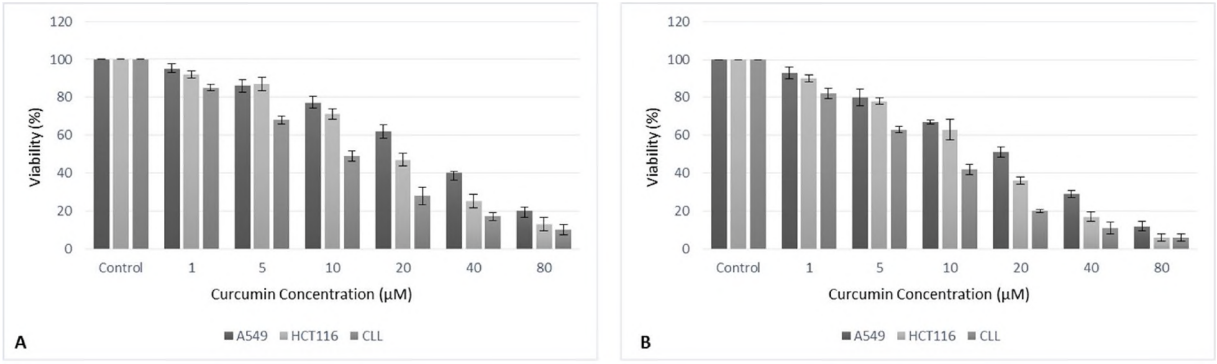

Fig. 1. Effect of curcumin on the viability of A549, HCT116 and 183E95 cells after 12 h (A) and 24 h (B). The results are provided as mean values with standard deviations from at least three independent experiments. Values were compared to the control and values with $p < 0.05$ were considered statistically significant.

3.2. Curcumin down-regulated expression of CD44 time- and dose-dependently in cancer cells

The expression of CD44, as CSC marker, was evaluated in A549, HCT116 and 183E95 cells. The changes of CD44 expression under treatment with curcumin (1, 5, 10, 20, 40 and 80 µM) for 12 and 24 h in comparison with untreated control cells was determined. In the flow-cytometric analysis, the percentage of CD44 expression was 61.77 ± 5.07, 1.44 ± 0.36, and 24.83 ± 1.93 in A549, HCT116 and 183E95 cell lines, respectively (Table 1). Followed by treatment with curcumin, CD44 expression significantly decreased in a concentration dependent manner in all investigated cell lines (Table 1 and 2). Expression of CD44 decreased by 90 % after 12 h treatment in 183E95 cells already at a dosage of 1 µM curcumin. In HCT116 and A549 cells 10 µM and 20 µM curcumin respectively were needed to suppress 90 % of CD44 expression.

3.3. CD44, CD133 and ALDH1 were time-dependently suppressed by curcumin in colon cancer cells

Further, the expression of CD44, CD133 and ALDH1 were analyzed exemplarily in the cell line HCT166 using immunofluorescence and

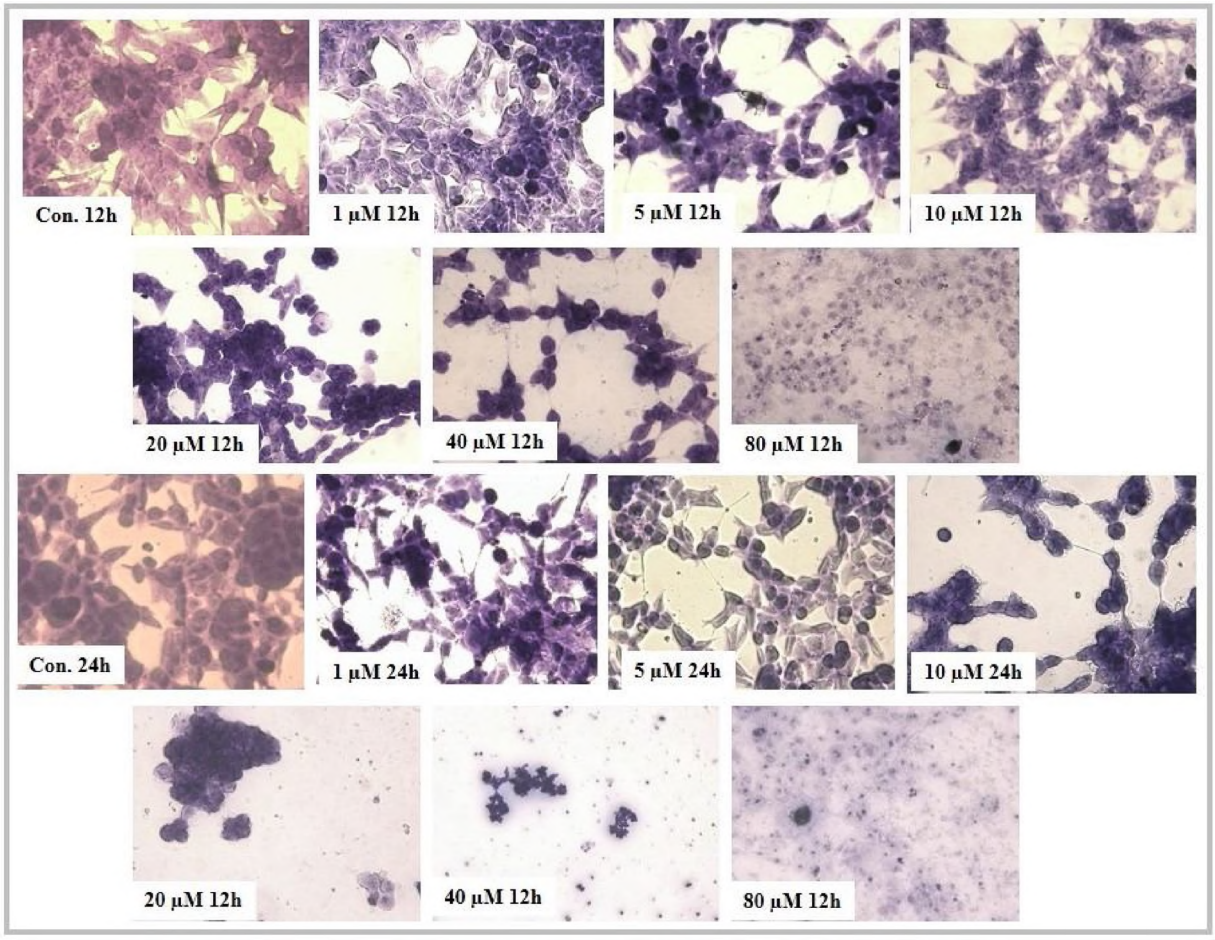

Fig. 2. The monolayer cultures of HCT116 cells were either left untreated or were treated with different concentration of curcumin (1 µM, 5 µM, 10 µM, 20 µM, 40 µM and 80 µM) for 12 and 24 h respectively. The cells showed concentration dependent morphological chage and growth inhibition.

Table 1
Effect of curcumin on the expression of CD44 in A549, HCT116 and 183E95 cells after 12 h. Flowcytometry analysis was performed in triplicate and data are shown as mean values with standard deviations from at least three independent experiments. Values were compared to the control and values with $p < 0.05$ were considered statistically significant.

	CD44 EXPRESSION (%) IN TESTED CELL LINES (12 hrs. after treatment)		
CURCUMIN CONCENTRATION (μM)	**A549**	**HCT116**	**183E95**
Control	61.77 ± 5.08	1.44 ± 0.32	24.83 ± 1.93
1	50.16 ± 1.21	0.54 ± 0.03	1.69 ± 0.06
5	25.12 ± 1.33	0.44 ± 0.04	1.46 ± 0.04
10	10.14 ± 0.02	0.18 ± 0.05	1.45 ± 0.06
20	1.11 ± 0.01	0.11 ± 0.01	1.07 ± 0.16
40	0.42 ± 0.11	0.10 ± 0.02	0.97 ± 0.07
80	0.31 ± 0.03	0.05 ± 0.02	0.66 ± 0.10

Table 2
Effect of curcumin on the expression of CD44 in A549, HCT116 and 183E95 cells after 24 h. Flowcytometry analysis was performed in triplicate and data are shown as mean values with standard deviations from at least three independent experiments. Values were compared to the control and values with $p < 0.05$ were considered statistically significant.

	CD44 EXPRESSION (%) IN TESTED CELL LINES (24 hrs. after treatment)		
CURCUMIN CONCENTRATION (μM)	**A549**	**HCT116**	**183E95**
Control	61.77 ± 5.08	1.44 ± 0.32	24.83 ± 1.74
1	22.58 ± 2.83	0.33 ± 0.08	2.23 ± 0.19
5	6.82 ± 1.29	0.16 ± 0.03	1.69 ± 0.17
10	2.06 ± 0.27	0.12 ± 0.02	1.28 ± 0.20
20	1.38 ± 0.41	0.09 ± 0.01	1.31 ± 0.13
40	0.09 ± 0.01	0.04 ± 0.01	1.11 ± 0.18
80	0.03 ± 0.03	0.01 ± 0.02	0.22 ± 0.03

western blot technique. Immunofluorescence staining showed clearly that the expression of the mentioned CSC markers were down-regulated within 60 min (Fig. 3). Interestingly, the western blot analysis (Fig. 4) revealed that the expression of CD44, CD133 as well as ALDH1 were down regulated time dependent even with low concentration of curcumin (5 μM), which denoted that curcumin not only decreased the viability of the cancer cells but also down regulated the CSC markers.

3.4. Addition of FCS to the culture medium stabilized curcumin and delayed degradation

In the present study, a remarkable correlation between decrease of CD44 and duration of experiment (12 and 24 h) in any of the tested cell lines could not be found (Table 1 and 2). Hence, to determine the stability of curcumin, we checked curcumin concentration in four different culture systems analyzing exemplarily the cell line A549 (RPMI-1640, RPMI-1640 + 10 % FCS, RPMI-1640 + A549 cells, RPMI-1640 + 10 % FCS + A549 cells). Curcumin was added to each medium culture to achieve a final concentration of 80 μM. The curcumin concentration in the supernatant of each culture system was measured every three hours for 24 h by using the HPLC method as described in detail in Materials and Methods. Comparison of the total curcumin concentration at each time point to the initial value showed significant reduction in a time-dependent manner in each culture system (Table 3). Interestingly, it was observed that curcumin underwent rapid degradation without FCS supplementation and was reduced to a concentration of approximately 16 μM after the first six hours independent of cells in the culture. Adding 10 % FCS increased curcumin stability so that the concentration of curcumin remained approximately 67 and 58 μM, respectively, in the absence or presence of A549 cells after the first six hours (Table 3). After 24 h, more than 90 % of total curcumin was degraded in the medium without FCS, whereas adding 10 % FCS could markedly stabilize 50–55 % of curcumin. To investigate in more detail which component of curcumin was the most stable in culture medium HPLC was performed for individual curcumin and curcuminoids. The HPLC analysis of total curcumin revealed three separate peaks with different retention times corresponding to curcumin (diferuloylmethane; curcumin I), desmetoxycurcumin (p-hydroxycinnamoylferuloyl-methane, curcumin II) and bisdemetoxycurcumin (di-p-hydroxycinnamoylmethane, curcumin III) (Fig. 5A). The HPLC profiles of the three curcuminioid concentrations were estimated in each culture system (Table 4). After the first six hours, ~90 % reduction in CUR I, ~40 % reduction in CUR II and ~16 % reduction in CUR III were observed in medium without FCS (Table 4 and Fig. 5B and C). Further, ~17 reduction of CUR I, ~2 % of CUR II and <1 % of CUR III were observed in medium supplemented with 10 % FCS alone (Table 4 and Fig. 5D) and ~27 %, ~19 % and ~13 % reduction of CUR I, CUR II and CUR III were observed in the presence of 10 % FCS and cells respectively (Table 4 and Fig. 5E). Although addition of 10 % FCS significantly increased stability in all three forms of curcuminoids especially in CUR I, the presence of the cells in the culture systems had no significant effect on the degradation of curcumin. Overall, the experiments showed that 10 % FCS supplementation could stabilize curcumin in the culture medium. In addition, it was illustrated that the lowest and the highest stability was found for CUR I and CUR III, respectively.

4. Discussion

Lung and colon cancers are ranked among the most common types of malignancies resulting in death worldwide (Jemal et al., 2011). Moreover, leukemia is between the ten most common cancer-related death and chronic lymphocytic leukemia is the most frequent leukemia in adults for which therapeutic options remain unsatisfying (Balducci and Dolan, 2015; Jemal et al., 2011). In order to overcome the limitations of standard treatment protocols, phytochemicals from traditional medical plants are being widely examined for their beneficial roles against cancers (Buhrmann et al., 2016).

In this study the potential suppressive effect of the natural polyphenol curcumin on the expression of cancer stem cell markers CD44, CD133 and ALDH1 in various cancer cell lines derived from colon cancer (HCT116), lung cancer (A549) and chronic lymphocytic leukemia (183E95) was investigated. Additionally, as the bioavailability is a limiting factor for treatment potential of natural agents, the authors examined the stability of curcumin in medium and/or cell culture supplemented with or without 10 % FCS.

It was demonstrated with a MTT viability and proliferation assay that curcumin significantly suppressed viability and proliferation in all three cell lines in a time- and dose-dependent manner. The growth inhibition could be also demonstrated with Giemsa staining. The polyphenol curcumin is a biologically active natural component derived from the plant turmeric (curcuma longa) which can induce cancer cell death through triggering a series of intracellular pathways related to apoptotic processes (Ravindran et al., 2009; Shakibaei et al., 2007, 2013; Toden et al., 2015). The results are in line with studies that have supported the cytotoxic effect of curcumin in a dose- and time- dependent manner in various cancer cell lines (Collett and Campbell, 2004; Pillai et al., 2004). It was found that overall, lung cancer cells A549 required higher and lymphocytic cells 183E95 lower dosages of curcumin to suppress proliferation and viability compared to colon cancer cells HCT116. Indeed, it has been previously reported that the

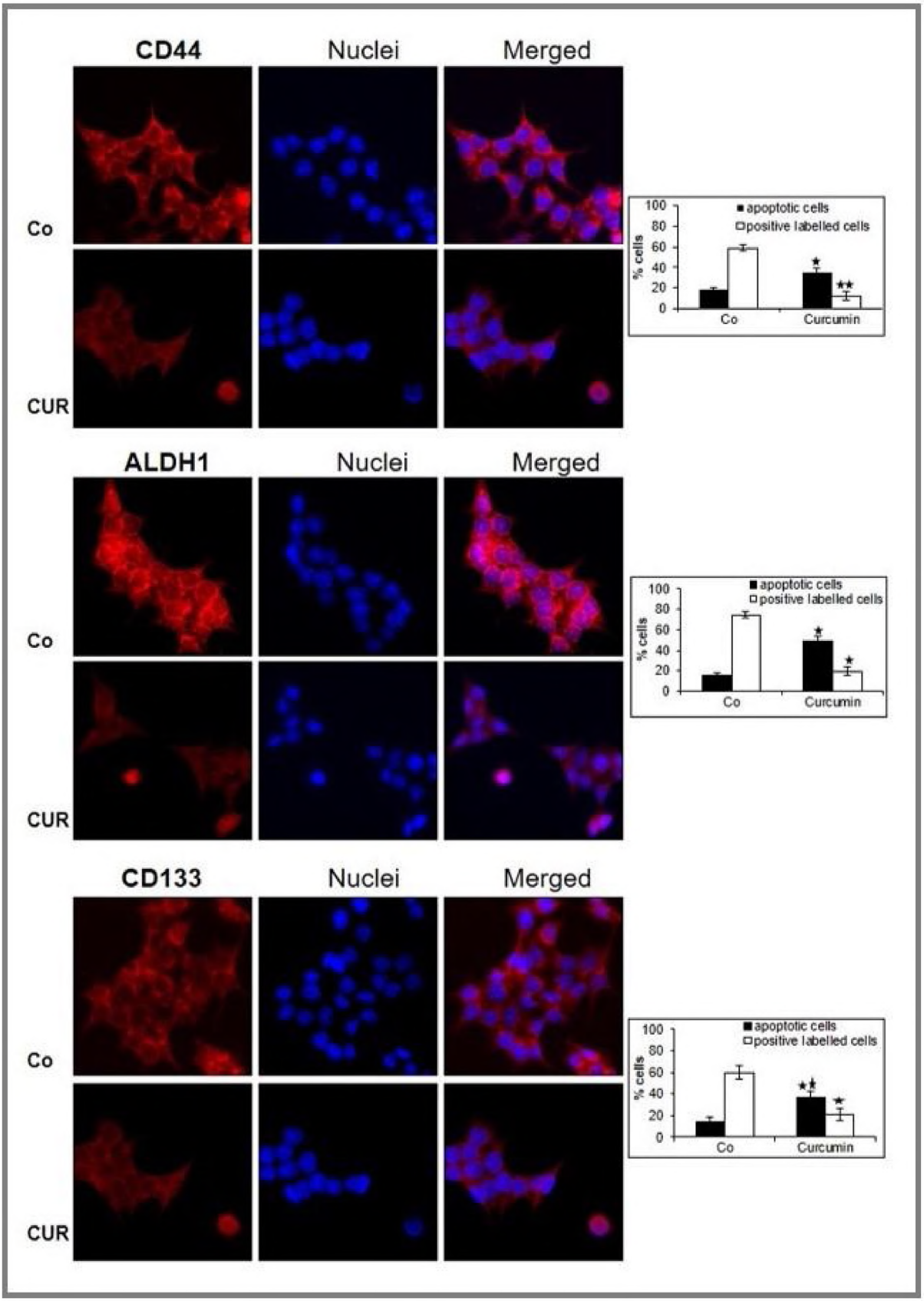

Fig. 3. Colon cancer stem cell marker expression in monolayer cultures as shown by immunofluorescence labeling. Monolayer cultures of HCT116 cells were either left untreated or were treated with curcumin (5 μM) for 60 min. The cells were subjected to immunofluorescence labelling with primary antibodies against CD44, CD133 and ALDH1, followed by incubation with rhodamine-coupled secondary antibodies and counterstaining with DAPI to visualize cell nuclei. Pictures shown are representative of three different experiments. Magnification 600x; Scale = 30 μm.

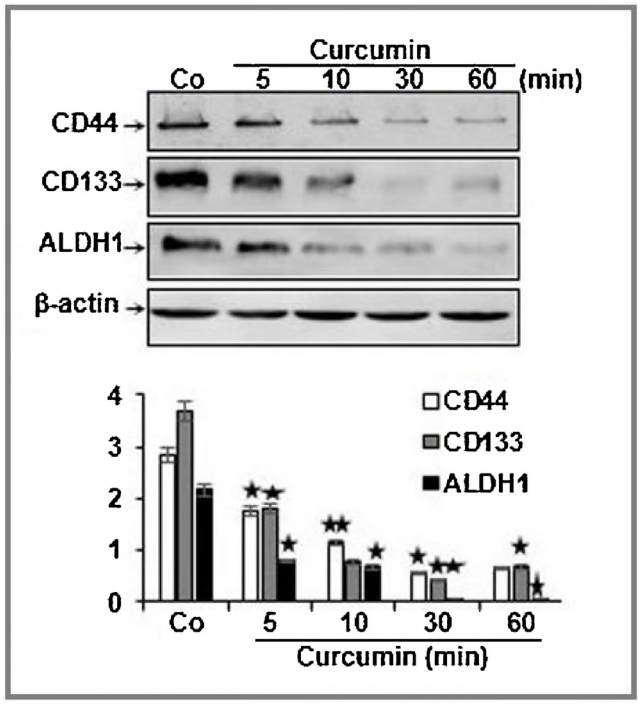

Fig. 4. Effects of curcumin on cancer stem cell marker expression in HCT116 cells in monolayer culture as shown by western blotting evaluation. HCT116 cells in monolayer culture were treated as described in Materials and Methods. Immunoblotting of whole cell lysates was performed for anti-CD133, -CD44 and -ALDH1. The results are shown from at least three independent experiments and the housekeeping protein β-actin served as an internal loading control. Densitometric evaluation was performed for CD133, CD44 and ALDH1. *P < 0.05, **P < 0.01.

dosage to induce cytotoxic effects of curcumin on cancer cells varied between the investigated cell lines (Perrone et al., 2015; Taverna et al., 2015; Ali et al., 2017). Further, these results are consistent with results in colon cancer cells obtained by Shakibaei et al. (2013, 2014) that curcumin alone or in combination with 5-FU significantly blocked cell proliferation, formation of colonospheres and induced apoptosis. Furthermore, Everett et al. reported that the mean IC50 of curcumin in normal mononuclear blood cells was 21.8 μM, whereas this value was 5.5 μM for B cells obtained from chronic lymphocytic leukemia patients (Everett et al., 2007). This emphasizes the multi-targeting potential of curcumin and its low toxicity to normal and healthy cells.

It is widely recognized today that there is a small fraction of cancer cells, which exhibit stem cell characteristics (Kreso and Dick, 2014). These cancer stem cells (CSCs) are responsible for tumor chemoresistance, recurrence and tumor metastases (Jiao et al., 2016; Jordan et al., 2006; Ricci-Vitiani et al., 2009; Kozovska et al., 2014). Indeed, CSCs show higher survival, pluripotency, are highly proliferative and exhibit strong invasion and migration properties (Kreso and Dick, 2014; Boral and Nie, 2012). The surface markers CD44 and CD133 and the enzyme Aldehyde dehydrogenase 1 (ALDH1) have been described as markers for CSCs in colon cancer, lung cancer and leukemia and high expression has been associated with increased tumor growth, metastasis and poorprognosis (Kozovska et al., 2014; Leon et al., 2016; Fonseca et al., 20180.

With Flowcytometry it was found that in untreated controls cancer stem cell marker CD44 was differently expressed in all three investigated cell lines: 61.77 ± 5.07 % in A549, 1.44 ± 0.36 % in HCT116 and 24.83 ± 1.93 in 183E95 cells. Reasons for this may be found in inter-individual differences in each cell line, in structural and functional differences of cells and further in variation of laboratory and clinical settings during experiments. Indeed, CD44 positive cancer cells have been found to range between 0–100 percent in different cell lines (Stuelten et al., 2010). There are even differences between CD44 expression in cells within a cell line as reported by several research groups (Stuelten et al., 2010; Wang et al., 2012; Leung et al., 2010).

Studies have shown that CSCs are majorly responsible for failure of treatment strategies as they promote tumor chemoresistance, underlining that for successful treatment strategies, elimination of CSCs is vital (Abdullah and Chow, 2013; Baumann et al., 2008). Indeed, novel treatment strategies that specifically target CSCs are very promising as future treatment strategies for managing cancer (Ajani et al., 2015; Wang et al., 2014). Interestingly the majority of the described CSC markers are also found in embryonic and adult normal stem cells, underlining their physiological importance for cellular self-renewal (Kim and Ryu, 2017).

Therefore, next the effect of curcumin on suppressing CSC marker expression in cancer cell lines with Flowcytometry was investigated. The results demonstrated that curcumin significantly suppressed CD44 expression in a dose- and time- dependent manner in all three investigated cell lines. Interestingly, CD44 expression in the chronic lymphocytic leukemia cell line (183E95) was much more sensitive to curcumin treatment. Here significant suppression of CD44 was observed already with 1μM curcumin. A reason for this may be found in the mutli-targeting effect of curcumin *via* CSC self-renewal pathways and specific microRNAs involved in acquisition of epithelial-mesenchymal transition (EMT) may be additionally blocked leading to enhanced suppression of CD44 (Li and Zhang, 2014).

Further, the authors investigated the colon cancer cell line HCT166 exemplarily for CD44, CD133 and ALDH1 expression with immunofluorescence and western blot analysis. It was shown clearly that even low dosage of curcumin (5μM) significantly down regulated the expression of CSC biomarkers CD44, CD133 and ALDH1 dose-dependently. As reported by many researchers, curcumin has a significant ability to influence CSC expression and modulate intercellular signaling pathways in many cancer cell lines (Shakibaei et al., 2014; Li and

Table 3
Stability of curcumin in four different culture systems. The results are provided as mean values with standard deviations from at least three independent experiments. Values were compared to the control and values with p < 0.05 were considered statistically significant.

	CURCUMIN CONCENTRATION IN FOUR DIFFERENT CULTURE SYSTEMS (μM)			
TIME AFTER TREATMENT (hrs.)	**MEDIUM**	**MEDIUM + CELLS**	**MEDIUM + 10 % FCS**	**MEDIUM + 10 % FCS + CELLS**
0	80.00 ± 1.07	80.00 ± 1.13	80.00 ± 1.49	80.00 ± 0.96
3	28.24 ± 1.04	21.70 ± 2.14	73.09 ± 1.63	64.70 ± 1.55
6	16.05 ± 0.57	16.82 ± 0.39	67.45 ± 1.60	58.68 ± 1.80
9	13.79 ± 1.10	12.51 ± 0.75	61.95 ± 1.24	50.56 ± 0.56
12	11.73 ± 1.14	10.02 ± 0.58	57.86 ± 0.91	46.59 ± 1.33
15	10.66 ± 0.51	9.66 ± 0.52	54.39 ± 0.41	45.57 ± 2.12
18	10.43 ± 0.53	8.88 ± 0.59	50.49 ± 2.38	42.84 ± 2.12
21	9.47 ± 0.43	8.44 ± 0.61	47.12 ± 0.54	36.28 ± 0.96
24	7.08 ± 0.81	8.31 ± 0.25	44.05 ± 2.77	34.48 ± 0.98

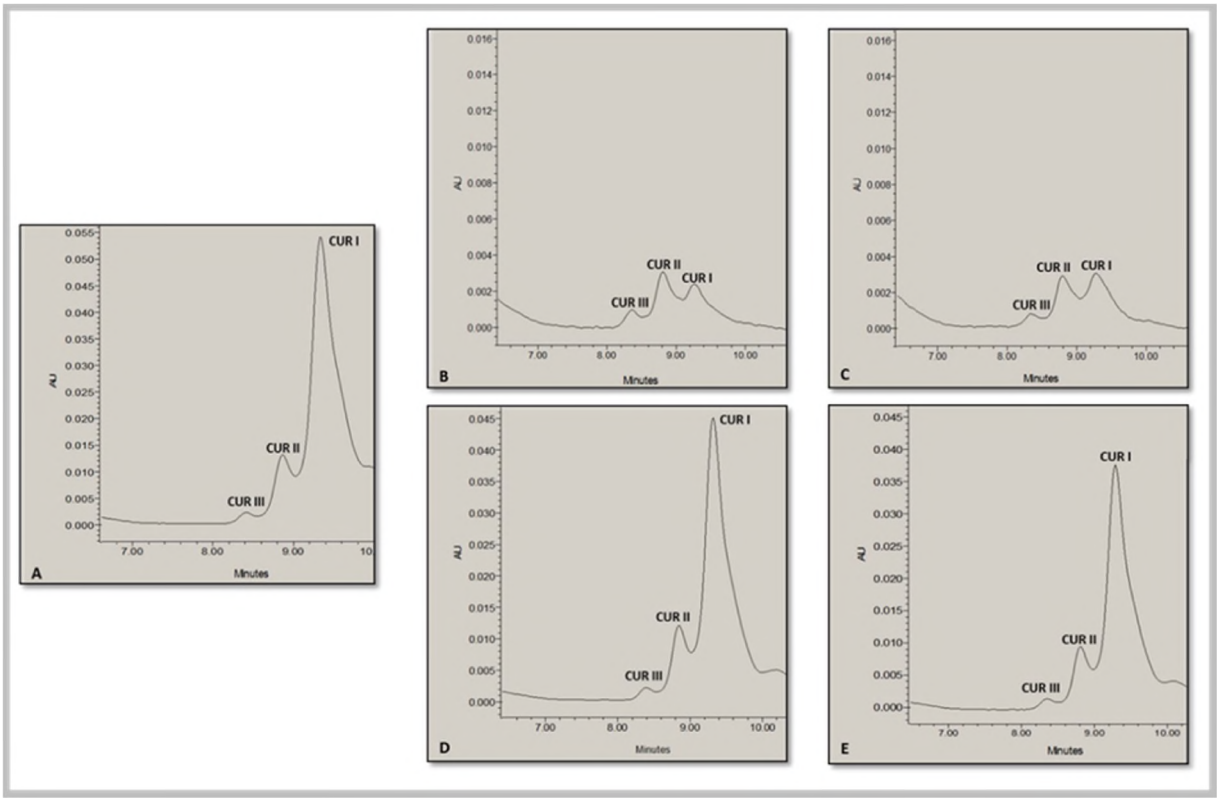

Fig. 5. HPLC analysis of curcumin revealed three peaks attributed to curcumin I, II and III (A). HPLC profile of curcumin I, curcumin II and curcumin III in RPMI-1640 (B), in RPMI-1640 + A549 cells (C), in RPMI-1640 + 10 % FCS (D) and in RPMI-1640 + 10 % FCS + A549 cells (E).

Zhang, 2014; Buhrmann et al., 2014), thereby being very promising for novel therapeutic approaches in cancer. Indeed, Shakibaei et al. (2014) showed the strong chemosensitizing effect of curcumin on colon CSCs and down-regulation of colon CSC markers followed by curcumin treatment alone or in combination with 5-FU. Similar results have been reported by Yu et al. (2009) in which curcumin alone or together with FOLFOX resulted in a notable reduction in CSCs, as evidenced by the decreased expression of CD44, CD166 and EGFR and also by their decreased ability to form anchorage-dependent colonies. These results markedly highlight the potential of curcumin as a natural product for potential therapeutic applications in specifically targeting cancer stem cells.

Bioavailability of curcumin is a basis for successful cancer treatment. Therefore, stability of curcumin in different conditions was investigated. The rate of curcumin decrease dramatically accelerated in FCS-free medium compared to medium supplemented with 10 % FCS. Interestingly, the presence of cells did not significantly influence changes in curcumin concentration in the above-mentioned mediums. Although much research has been performed over the last decades which confirmed the therapeutic effectiveness of curcumin in cancer and other inflammation-related diseases, its rapid degradation or poor bioavailability are a major hurdle for clinical application (Anand et al., 2007). A variety of factors have been reported to affect chemical stability of dissolved curcumin such as pH, solvent, oxygen, ultraviolet waves, visible light and presence of metal ions (Metzler et al., 2013). Indeed, lowering the pH, addition of ascorbic acid, N-acetyl cysteine and glutathione in culture media and/or complex-formation with cyclodextrin may increase the stability of curcumin (Wang et al., 1997; Oetari et al., 1996; Tomren et al., 2007). In addition, Low solubility, poor absorption, low intrinsic activity, rapid metabolism, inactivity of metabolic products and rapid systemic elimination, which mainly result from auto-oxidative transformation, can seriously limit curcumin application (Anand et al., 2007; Schneider et al., 2015). The results are in line with a study from Pfeiffer et al. (2003) which showed that the stability of curcumin is affected in a time dependent fashion and that addition of serum into the culture medium increased the stability of curcumin.

Further, importantly on lung cancer cells A549 it was demonstrated with HPLC that different components of curcumin displayed varying stability in cell culture medium. Commercially available curcumin is not a homogenous chemical compound but a mixture of three distinguished analogues: Curcumin (diferuloylmethane, curcumin I) (82 %), desmetoxycurcumin (p-hydroxycinnamoylferuloyl-methane, curcumin II) (15–17 %) and bisdemetoxycurcumin (di-p-hydroxycinnamoylmethane, curcumin III) (3–5 %) inclusively referred to as curcuminoids (Anand et al., 2008). Interestingly, it could be shown that 10 % FCS in the culture medium significantly increased stability of all three curcumin components. The most stable being curcumin III, intermediate stability curcumin II and the least stable curcumin I. These findings are in line with a previous study, demonstrating different stabilities for the three curcuminoids (Pfeiffer et al., 2003). Indeed, it has been shown that the mixture of these curcuminoids may have a more powerful intracellular effect than either of them singly (Ahmed and Gilani, 2009).

5. Conclusion

These findings underline the possible potential of the natural agent curcumin for clinical applications by also targeting cancer stem cells. The stability of curcumin and curcuminoid components was markedly prolonged in medium supplemented with 10 % FCS, however the low aqueous solubility and bioavailability of curcumin in the formulation used for this study is a limitation. To overcome this problem more research needs to be conducted. Additionally more research needs to be conducted to see if this formulation is useful in clinical conditions.

Table 4
Stability of curcumin I (CUR I), curcumin II (CUR II) and curcumin III (CUR III) in four different culture systems. The results are provided as mean values with standard deviations from at least three independent experiments. Values were compared to the control and values with $p < 0.05$ were considered statistically significant.

	CURCUMINOIDS CONCENTRATION IN FOUR DIFFERENT CULTURE SYSTEMS (μM)											
TIME AFTER TREATMENT (hrs.)	**MEDIUM**			**MEDIUM + CELLS**			**MEDIUM + 10 % FCS**			**MEDIUM + 10 % FCS + CELLS**		
	CUR I	**CUR II**	**CUR III**	**CUR I**	**CUR II**	**CUR III**	**CUR I**	**CUR II**	**CUR III**	**CUR I**	**CUR II**	**CUR III**
0	64.94 ± 0.43	10.64 ± 0.29	4.42 ± 0.41	65.90 ± 1.02	10.51 ± 0.49	3.59 ± 0.45	68.92 ± 1.48	9.84 ± 0.49	1.24 ± 0.09	68.85 ± 0.67	9.87 ± 0.32	1.28 ± 0.08
3	16.28 ± 2.30	8.14 ± 0.95	3.82 ± 0.72	11.46 ± 1.12	6.76 ± 0.81	3.48 ± 0.40	62.11 ± 1.51	9.75 ± 0.17	1.23 ± 0.11	54.88 ± 0.97	8.69 ± 0.53	1.13 ± 0.11
6	6.01 ± 1.70	6.36 ± 0.81	3.68 ± 0.58	7.71 ± 0.33	5.83 ± 0.37	3.28 ± 0.35	56.69 ± 1.46	9.59 ± 0.39	1.17 ± 0.09	49.76 ± 0.97	7.94 ± 0.86	1.11 ± 0.02
9	5.24 ± 0.48	5.18 ± 0.57	3.37 ± 0.38	4.18 ± 0.33	5.17 ± 0.42	3.16 ± 0.52	51.70 ± 0.61	9.10 ± 0.64	1.15 ± 0.12	42.28 ± 0.49	7.33 ± 0.22	0.95 ± 0.06
12	4.02 ± 0.61	4.33 ± 0.41	3.38 ± 0.28	2.79 ± 0.79	4.12 ± 0.32	3.11 ± 0.34	47.67 ± 0.29	9.06 ± 0.87	1.13 ± 0.24	38.72 ± 0.78	7.07 ± 0.61	0.80 ± 0.07
15	3.65 ± 0.33	3.74 ± 0.36	3.27 ± 0.24	2.71 ± 0.45	3.89 ± 0.41	3.06 ± 0.44	44.29 ± 0.81	8.97 ± 0.58	1.13 ± 0.05	38.21 ± 1.73	6.57 ± 0.41	0.79 ± 0.04
18	3.54 ± 0.35	3.69 ± 0.33	3.20 ± 0.34	2.65 ± 0.32	3.22 ± 0.36	3.01 ± 0.32	40.57 ± 1.71	8.85 ± 0.64	1.07 ± 0.07	36.26 ± 1.80	5.86 ± 0.46	0.72 ± 0.09
21	3.33 ± 0.78	2.99 ± 0.82	3.15 ± 0.39	2.58 ± 0.40	2.98 ± 0.57	2.88 ± 0.24	37.27 ± 0.36	8.79 ± 0.78	1.06 ± 0.05	30.06 ± 1.02	5.50 ± 0.24	0.72 ± 0.08
24	1.62 ± 0.72	2.40 ± 0.81	3.06 ± 0.53	2.52 ± 0.77	2.95 ± 0.38	2.84 ± 0.41	34.22 ± 2.30	8.77 ± 0.76	1.06 ± 0.12	28.51 ± 1.30	5.41 ± 0.49	0.56 ± 0.03

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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