Effects of green tea, matcha tea and their components epigallocatechin gallate and quercetin on MCF-7 and MDA-MB-231 breast carcinoma cells

LENNARD SCHRÖDER[1*], PHILIP MARAHRENS[2*], JULIAN G. KOCH[1], HELENE HEIDEGGER[1], THERESA VILSMEIER[1], THUY PHAN-BREHM[1], SIMONE HOFMANN[1], SVEN MAHNER1, UDO JESCHKE[1] and DAGMAR U. RICHTER[2]

[1]Department of Obstetrics and Gynecology, University Hospital, Ludwig Maximilians University (LMU) of Munich, D-80337 Munich; [2]Department of Obstetrics and Gynecology, University of Rostock, D-18059 Rostock, Germany

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Abstract. We investigated the anticarcinogenic potential of green tea and its components epigallocatechin gallate (EGCG) and quercetin, as well as tamoxifen, on MCF-7 and MDA-MB-23 breast cancer cells. Using high-performance liquid chromatography, the quantity of EGCG and quercetin in green tea was analyzed. The receptor status of the cells was confirmed immunohistochemically. Various viability and cytotoxicity tests were later performed to investigate the effects of the substances. After incubating the cells with green tea extract, EGCG, quercetin and tamoxifen, a decrease in viability (MTT test) or proliferation (BrdU assay) was found in all cell tests with varying effects, depending on the assay used. The effects were similar in both cell lines. This work confirmed that EGCG and quercetin are contained in green tea and that both substances in pure form and as green tea have an anticarcinogenic effect on both estrogen receptor-positive and -negative breast cancer cells. This effect could also be demonstrated with tamoxifen in both cell lines (MTT and BrdU assays). These results suggest that the effects observed in these experiments are not generated only via estrogen receptor-mediated pathways.

Introduction

After water, tea is the most commonly consumed beverage worldwide (1). Even in Western countries, such as Germany, tea consumption is increasing. In 2015, an average consumption of 28 liters per capita was registered, corresponding to approximately 20,000 tons of total domestic tea consumption. Since 2005, the share of green tea (2005: 20%) against black tea (2005: 80%) has increased to 30% (2). Similar to black tea, green tea is made from the leaves of the tea plant *Camellia sinensis* (1).

Recently, matcha tea (MT) has been gaining in popularity. The substrate concentrations are higher than that in other green teas because the leaves are ground with a ceramic mill to a fine powder that is later dissolved in hot water and completely consumed. Fujioka *et al* published a study in 2016 in which they observed that the protective effect of MT against oxygen radicals is significantly higher than the effect of normal tea leaves due to increased catechin levels (3).

Research on polyphenols in green tea (GT) has revealed considerable health benefits (4). The polyphenol family includes the flavonoids which, in turn, incorporate flavanols and catechins. Approximately 30% by mass of the dry substance of fresh tea leaves is made up of phenolic compounds. In GT, 90% of these polyphenolic compounds are catechins. The most abundant catechin in GT is epigallocatechin gallate (EGCG) (5). Epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) are also catechins found in GT. However, these only make up 3-6% (EGC, ECG) and 1-3% (EC) of the dry mass. Other components of the tea leaves by percentage of dry mass are proteins (15%), amino acids (4%), caffeine (4%), raw fibers (26%), lipids (7%), other carbohydrates (7%), pigments, such as chlorophyll and carotenoids (2%) and minerals (5%) (6). A cup of GT (100 ml) contains approximately 20-100 mg of EGCG. The US Department of Agriculture states that 200 mg of EGCG are contained in 100 g of boiled GT (7,8).

It was demonstrated in various studies that EGCG and quercetin exhibit anticancer activity. Besides steroid receptor (9) and PPARγ receptor (10) interaction, other assumed mechanisms of action include interaction with the PI3K/Akt/mTOR signaling pathway (11), VEGF (12), the the 67-kDa laminin receptor (67-LR) (13), p53, Bax protein (14) and Bcl-2 (15).

Correspondence to: Professor Udo Jeschke, Department of Obstetrics and Gynecology, University Hospital, Ludwig Maximilians University (LMU) of Munich, Maistrasse 11, D-80337 Munich, Germany
E-mail: udo.jeschke@med.uni-muenchen.de

*Contributed equally

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In the present study, we analyzed the effects of GT and MT, as well as their components EGCG and quercetin, on MCF-7 and MDA-MB-231 breast carcinoma cells.

Materials and methods

Breast cancer cell lines and preparation of the test substances. MCF-7 (HTB-22™; ATCC; American Type Culture Collection, Manassas, VA, USA) (ER^+, PR^+) and MDA-MB-231 (HTB-26™; ATCC) (ER^-, PR^-) cell lines were purchased commercially. Green tea (GT) was dissolved in water or 70% ethanol and was added to the cell culture at two different concentrations. One gram of the MT powder or coarsely ground Chinese tea leaves were weighed and were later dissolved in 10 ml of distilled water or 70% ethanol. The solutions were heated in a microwave (Discover SP; CEM Corp., Matthews, NC, USA), with the water extracts at 70˚C and 80 W for 20 min and the ethanol extracts at 60˚C and 80 W for 20 min. The extracts were subsequently centrifuged (1250 x g, 10 min), and the supernatant was later filtered and concentrated by evaporation to a third of the starting solution using a vacuum concentrator (Eppendorf Concentrator S301; Eppendorf, Hamburg, Germany) at 45˚C. The extracts were frozen at -80˚C for storage. Before application, the tea extracts were centrifuged (10.410 x g for 10 min) again after thawing.

EGCG was dissolved in distilled water and tested at seven different concentrations in the cell cultures. Quercetin was dissolved in 50% DMSO and tested at three different concentrations in the cell cultures. Tamoxifen and estradiol solutions were dissolved in 70% ethanol and examined at a single concentration.

High-performance liquid chromatography. To evaluate the concentration of EGCG and quercetin in GT and MT, high-performance liquid chromatography (HPLC) was performed. After starting the ClarityChrom® (Knauer GmbH, Berlin, Germany), the sample syringe was filled with 20 µl of the extract to be tested (GT, MT, quercetin and EGCG) and analyzed for 40 min. The results were analyzed using ClarityChrom software (version 1670-2). Since the concentrations of the active substances were known, the quantity of the pure substance contained in the tea extract could later be calculated by cross-multiplication.

ATP luminescence test. The ATP luminescence test assessed the viability of the cells at different substrate concentrations. Next, 100 µl/well cell suspensions was transferred to a 96-well plate for cell incubation for 24 h at 37˚C in 5% CO_2. After the addition of 1 µl/well of the extract, further cell incubation was performed for 48 h at 37˚C and 5% CO_2. The CellTiter-Glo (Promega, Mannheim, Germany) substrate was mixed with the CellTiter-Glo (Promega) buffer and 100 µl/well was added. After 10 min of incubation at room temperature, the cell culture-reagent mixtures were added to a non-transparent 96-well plate (to avoid light spill to adjacent wells) and were measured using a luminescence reader in the CellTiter-Glo program (Promega).

Proliferation measurement using the MTT assay. Cell proliferation was analyzed using MTT (Roche, Mannheim, Germany). After incubation of cells (500,000/ml) for 24 h in the absence or presence of different substrate concentrations, the MTT-labeled reagent was added at a final concentration of 0.5 mg/ml and was later incubated again for 4 h. During this time, the metabolically active cells transformed the yellow tetrazolium salt MTT to purple-colored formazan crystals. After adding the solubilization solution, the plates were incubated overnight in a humidified atmosphere at 37˚C. With a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA), the color intensity was measured at 570 mm using a reference wavelength of 650 nm.

BrdU proliferation assay. Cell proliferation was analyzed using a 5-bromo-2'-deoxyuridine (BrdU) labeling and detection kit (Roche). MCF7 and MDA-MB-23 cells (500,000/ml) were grown in 96-well tissue culture plates for 24 h in the absence or presence of different substrate concentrations. After labeling with BrdU for 3 h, the cells were fixed, and BrdU incorporation into DNA was measured by ELISA. Cellular proliferation inhibition was expressed relative to the controls (100%) ± SD.

Neutral Red uptake assay. Cellular viability was analyzed using the Neutral Red uptake assay (Sigma-Aldrich, Steinheim, Germany). Next, 100 µl/well cell suspension was transferred to a 96-well plate for cell incubation for 24 h at 37˚C in 5% CO_2. After the addition of 1 µl/well extract, further cell incubation was performed for 24 h at 37˚C in 5% CO_2. The medium was replaced with Dulbecco's modified Eagle's medium (DMEM) without additives (pH adjusted to 7.2) and 3% Neutral Red test reagent was added. After 60 min of incubation at 37˚C in 5% CO_2, the medium was removed, and the wells were rinsed with 200 µl/well of phosphate-buffered saline (PBS). Next, 100 µl/well of Neutral Red solution was added, and the wells were placed on a plate vibrator. Finally, the wells were measured using an ELISA reader at 570 nm using a reference wavelength of 655 nm.

pH measurement. The pH was measured using a glass electrode. The electrode was calibrated before each measurement series.

Oxidative stress. The hydrogen peroxide colorimetric/fluorometric assay kit (BioVision Inc., Milpitas, CA, USA) was used to determine whether free oxygen radicals (ROS, reactive oxygen species) were produced. The quantity of hydrogen peroxide (H_2O_2) produced was determined photometrically, with the concentration of H_2O_2 being proportional to the measured optical density. After incubation, OxiRed™ (LinRed IHC, Dossenheim, Germany) was dissolved in 220 µl of DMSO (Sigma-Aldrich) and horseradish peroxidase (LinRed IHC, HRP) in 220 µl of H_2O_2-containing assay buffer. Next, 50 µl of the cell culture supernatant was pipetted on 96-well plates, and a H_2O_2 dilution series with concentrations of 0, 1, 2, 3, 4 and 5 nmol/well H_2O_2 was prepared. Fifty microliters of reaction mixture (48 µl of H_2O_2-containing assay buffer + 1 µl of HRP solution + 1 µl of OxiRed™ solution, LinRed IHC) was added. The samples were vortexed and incubated for 10 min at room temperature before undergoing measurement using an ELISA reader at 570 nm.

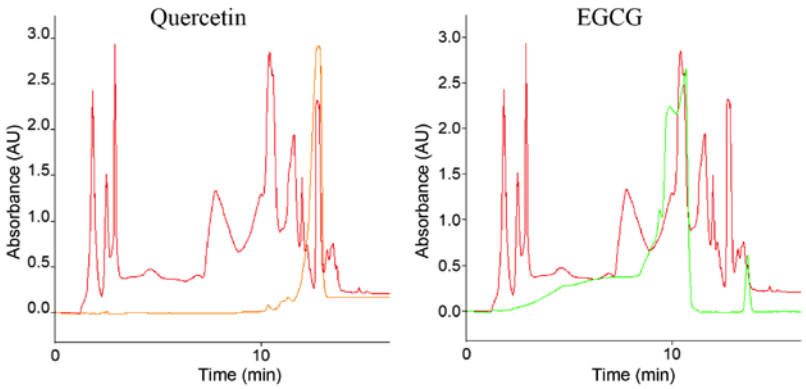

Figure 1. HPLC results in AU/min (ordinate = Absorbance in absorbance unit (AU), abscissa = time in min). MT dissolved in 70% ethanol (red), quercetin dissolved in 50% DMSO (orange) and MT dissolved in 70% ethanol (red), EGCG dissolved in aquadest (green). MT, matcha tea; EGCG, epigallocatechin gallate; HPLC, high-performance liquid chromatography.

Theory/calculation. The results were tested for normal distribution using the Kolmogorov-Smirnov test. Subsequently, one-factor variance analysis (ANOVA, analysis of variance) was carried out using with Bonferroni's post-hoc test. The statistics program IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA) was used for these analyses. A significance level of $\alpha = 0.05$ and a confidence interval of 95% were selected. Significant differences were examined between the respective test results and negative controls with the corresponding solvent, as well as between the two cell lines. The calculation of the correlations took place for interval-scaled features using the Pearsonian dimension correlation coefficient with MS Excel 2010. A very high correlation was assumed to be r>0.9.

Table I. Concentrations of EGCG and quercetin in mg/ml in the tea extracts (0.3 g of tea/1 ml H_2O).

	EGCG	Quercetin
MT in 70%-ethanol	13.5	1
GT in 70%-ethanol	15.6	1.1
MT in H_2O	6	1.2
GT in H_2O	11	1.1
Mean	11.5	1.1

EGCG, epigallocatechin gallate; MT, matcha tea; GT, green tea.

Results

HPLC. For each tea extract and pure substance, three measurements were performed. Fig. 1 shows as an example the superimposed HPLC graph of one tea extract + one pure substance. Superimposed peaks represent similar retention times, assuming that the two covering peaks are the same substance.

Quercetin and EGCG could be detected in MT and in GT in ethanol, as well as dissolved in H_2O, using HPLC. The retention times for quercetin were 12.9 min in each case and 10.7 min for EGCG. The concentrations of EGCG and quercetin in the respective tea extracts were calculated based on the respective peak areas. The results given are the mean values of two test series. Table I demonstrates the obtained concentrations.

ATP luminescence test/CellTiter-Glo® test. Tea extracts exhibited a significant inhibitory effect on cell viability (CV) in MDA-MB-231 cells in ethanol and water (Fig. 2A). Cell viability (CV) was below 2% using tea extracts. Using the mixture of EGCG and quercetin (Mix), significant inhibition of cell viability occurred (CV=32%, SD=2.1). Quercetin alone did not reach the ED_{50} at all of the measured concentrations and inhibited cell viability only at 30 μg/ml (CV=61.3%; SD=2.8). Different EGCG concentrations revealed a dose-response relationship (correlation r=0.96, i.e., the amount at r>0.9). With increasing concentrations, the inhibition of cell viability increased significantly for all measurements. The concentrations of 180, 150, 120 and 90 μg/ml were above ED_{50}. Estradiol did not significantly alter cell viability (98.7%, SD=5.6). Tamoxifen significantly inhibited the cell viability of MDA-MB-231 cells above the ED_{50} (CV<1%).

The results using MCF-7 cells were similar to those using the MDA-MB-231 cell line (Fig. 2B). All tea extracts were above the ED_{50} (CV<2%), similar to the mixture of EGCG and quercetin (Mix) (VC=33.2%; SD=13.6). In contrast to MDA-MB-231 cells, quercetin demonstrated an inhibitory effect beginning at a concentration of 10 μg/ml (CV=81.5, SD=7.1). As in MDA-MB-231 cells, a dose-response relationship was observed for different EGCG concentrations (correlation r=0.97, i.e., the amount at r>0.97). At concentrations of 90 to 180 μg/ml, the ED_{50} was reached. At lower concentrations up to 45 μg/ml, significant results were obtained. Estradiol led to slight cell proliferation (CV=107.3%, SD=3.0), and tamoxifen caused strong significant inhibition (CV=0.2%, SD=0.0). Comparing the results of MDA-MB-231 and MCF-7 cells, a significant difference between the two cell lines was found only in the result for quercetin at 30 μg/ml.

MTT assay. Tea extracts dissolved in ethanol had an inhibitory effect on MDA-MB-231 cells in both solvents and all, besides those in water, were above the ED_{50} (GT Eth: CV=40.1%, SD=6.9; GT H_2O: CV=41.2%, SD=15.3; Matcha H_2O: CV=54.4%, SD=12.7 (Fig. 3A). The mixture (Mix) of quercetin

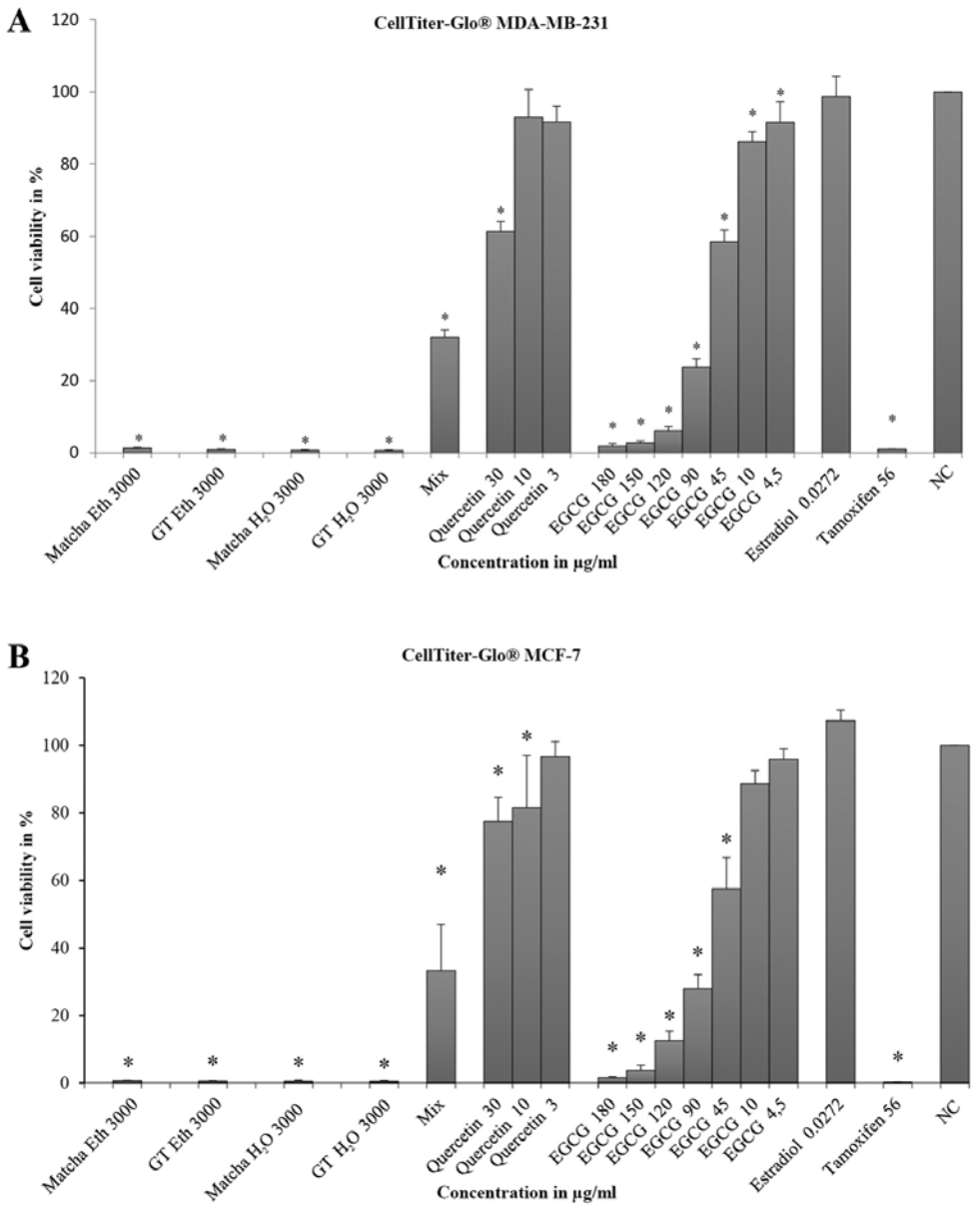

Figure 2. Measurement of adenosine triphosphate (ATP) with CellTiter-Glo® after 24 h in (A) MDA-MB-231 and (B) MCF-7 cells. Eth, dissolved in 70% ethanol; H_2O, dissolved in water; Mix, mixture of EGCG + quercetin, GT, green tea. The effects of quercetin and EGCG at different concentrations and estradiol and tamoxifen as controls on the cell viability relative to the negative control (NC) (100%). Mean values with positive standard deviations are represented. *P<0.05.

and EGCG significantly inhibited cell viability but did not reach the ED_{50} (CV=59.0%, SD=12.2). In the case of quercetin, a dose-response relationship was found, although statistical significance was only obtained at 30 μg/ml (CV=64.0%, SD=13.3). Although the cell viability was significantly inhibited at 45-180 μg/ml of EGCG, the ED_{50} could not be reached. Estradiol had no effect on cell viability compared with the negative control (CV=100.8%, SD=4.8) and tamoxifen was well above the ED_{50} (CV=2.3; SD=0.6).

In MCF-7 cells, tea extracts achieved significant inhibition of cell viability (>ED_{50}), (Matcha H_2O: CV=32.4% and SD=4.1; GT H_2O: CV=20.7% and SD=2.1; Matcha Eth: CV=47.1% and SD=2.6; GT Eth: CV=30.8% and SD=1.6) (Fig. 3B). The mixture (EGCG + quercetin = Mix) had little effect on cell viability (CV=91.4%, SD=2.0). Quercetin caused no significant inhibition of cell viability; 3 and 10 μg/ml fell above the value of the negative control (3 μg/ml: CV=116.6, SD=9.1, 10 μg/ml: CV=101.6, SD=5.3). Only at 30 μg/ml was minimal inhibition noticed (CV=99%, SD=7.6). EGCG only demonstrated significant inhibition of viability at 180 μg/ml (CV=66.0, SD=24.9). Concentrations between 45 and 4.5 μg/ml had no effect with CV values above 100%.

Comparing the MTT test results of MDA-MB-231 and MCF-7 cells, a significant difference between the two cell lines was found for quercetin at concentrations of 3 and 30 μg/ml and for EGCG at concentrations of 45 and 90 μg/ml.

BrdU assay. In MDA-MB-231 cells, significant inhibitory effects were only demonstrated using the mixture (EGCG + quercetin = Mix) (cell proliferation (CP)=51.9%, SD=16.8) and

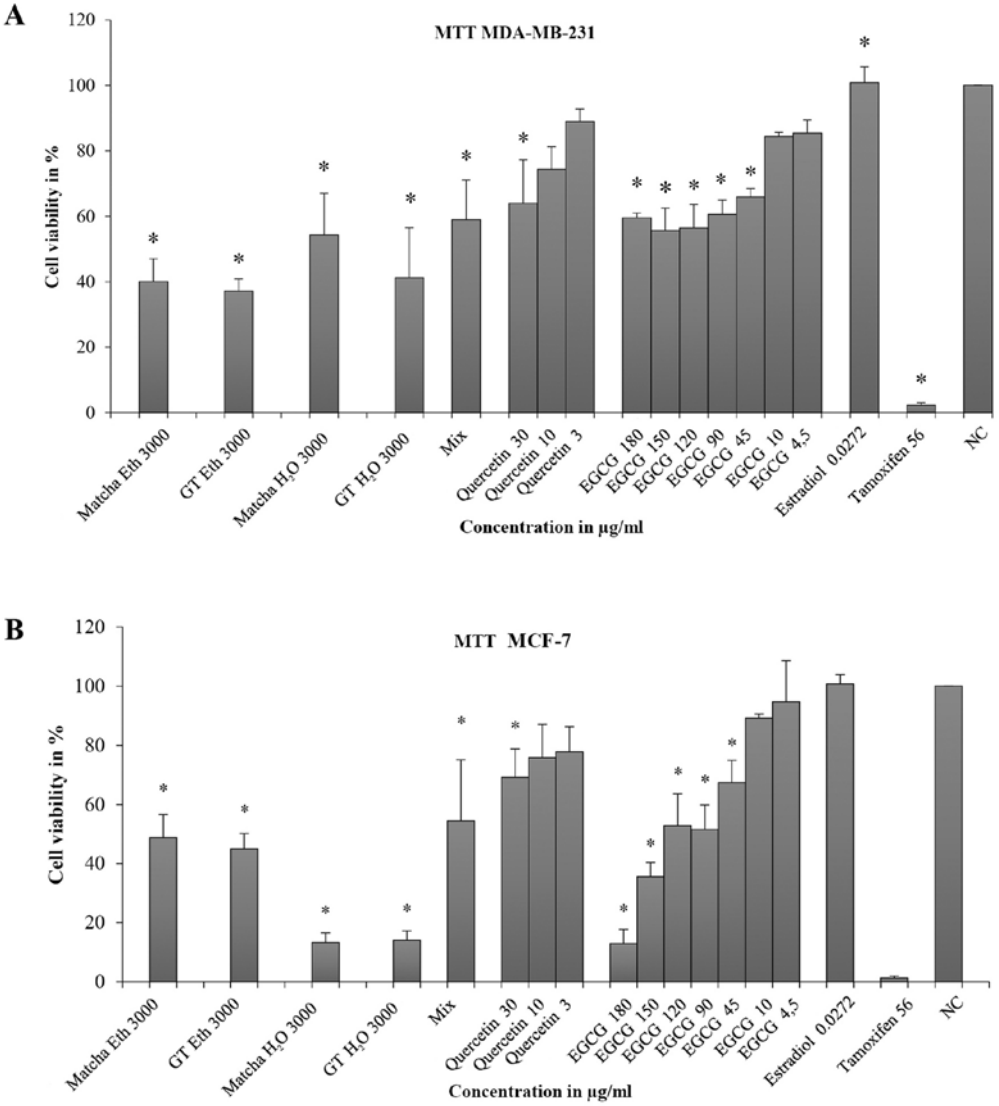

Figure 3. MTT test after 48 h in (A) MDA-MB-231 and (B) MCF-7 cells. (Eth = dissolved in 70% ethanol, H_2O = dissolved in water), mixture (EGCG + quercetin). The effects of quercetin and EGCG at different concentrations and of estradiol and tamoxifen as controls on the viability relative to the negative control (NC) (100%). The mean values with positive standard deviations are represented. *P<0.05.

tamoxifen (CP=1.0%; SD=0.2) (Fig. 4A). The ED_{50} was only reached using tamoxifen. The results for quercetin showed a dose-response relationship (30 μg/ml: CP=84.2%, SD=5.0; 10 μg/ml: CP=85.1%, SD=2.7; 3 μg/ml: CP=87.1%, SD=5.9). Estradiol showed a strong positive effect on cell proliferation (CP=0.71%, SD=0.23) compared with the negative control (NC) (CP=101.7%, SD=4.7).

In the BrdU test for MCF-7, the ED_{50} was not reached by any of the extracts except tamoxifen (CP=2.7%, SD=1.3) (Fig. 4B). At the lowest concentrations of quercetin (3 μg/ml: CP=105.2%, SD=2.7) and EGCG (4.5 μg/ml: CP=111.7%, SD=1.1) a proliferation-promoting effect was observed.

Neutral Red test. In MDA-MB-231 cells, a clear difference in the strength of inhibition between the tea extracts dissolved in ethanol or water was demonstrated (Fig. 5A). While the extracts dissolved in water showed strong inhibition of cell viability (CV) of 22.6% (SD=2.6) in GT and 25.8% (SD=4.1) in MT, ethanol extracts (Eth) only demonstrated a slight inhibition of 44.3% (SD=5.5) in GT and 55.1% (SD=7.2) in MTE. Statistical significance was achieved with all tea extracts. EGCG showed significant inhibition of cell viability at concentrations of 45-180 μg/ml, but the ED_{50} was reached only at 180 μg/ml.

In MCF-7 cells, as in the case of MDA-MB-231 cells, it was demonstrated that tea extracts dissolved in water (Matcha H_2O: CV=13.3%; SD=3.3; GT H_2O: CV=14.0%, SD=3.2) have a significantly more potent inhibitory effect on cell viability than when dissolved in ethanol (Matcha Eth: CV=45.0%, SD=5.2; GT Eth: CV=48.8%, SD=7.8) (Fig. 5B).

For EGCG, the ED_{50} was reached at 180 μg/ml (CV=13.0%, SD=4.8), 150 μg/ml (CV=35.5%, SD=4.8) and 90 μg/ml (CV=%, SD=7.1). Quercetin led to a significant inhibition of viability only at the highest concentration used (CV=69.2%, SD=9.5). None of the extracts led to an increase in viability (>100%); only the result for estradiol was similar to that for the negative control (NC) (CV=100.7%, SD=3.2). Tamoxifen treatment resulted in only 1.3% cell viability.

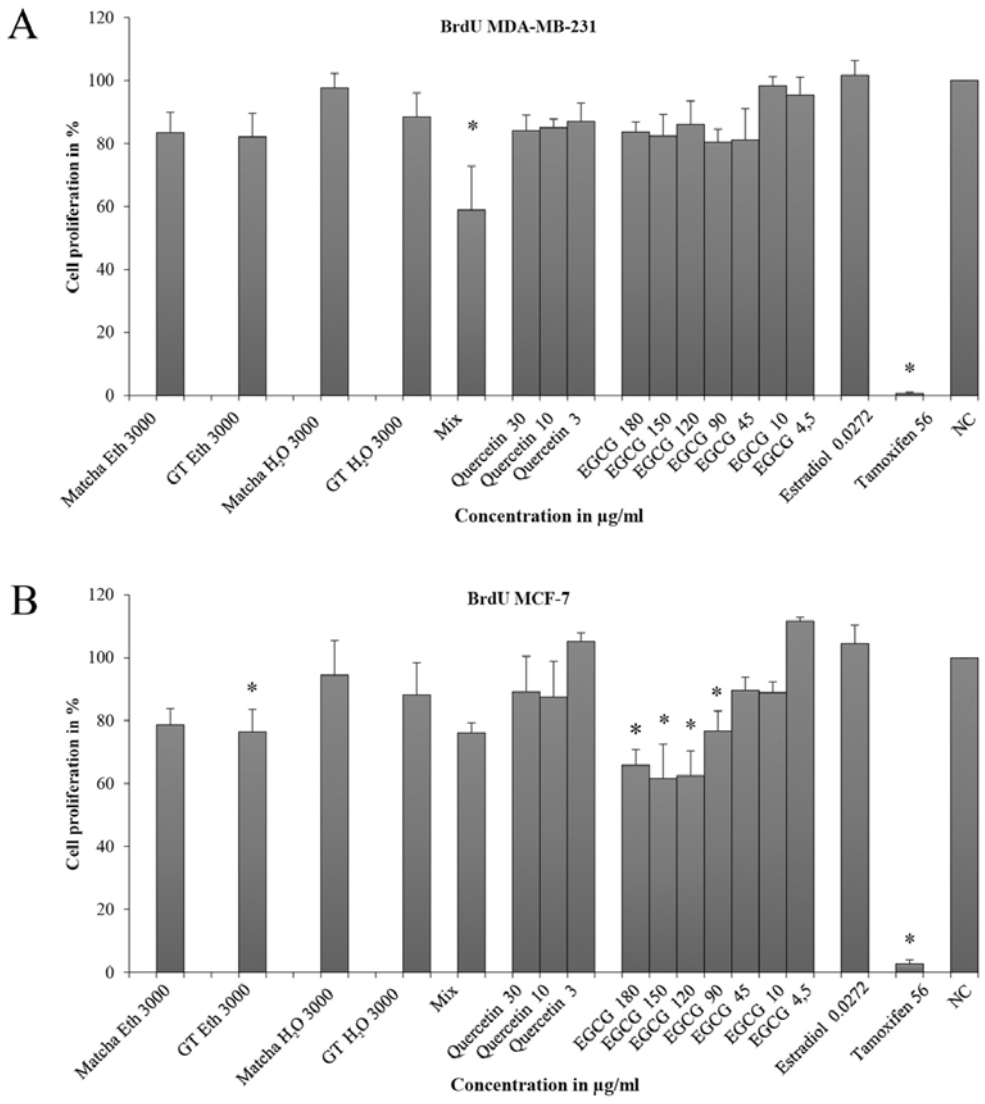

Figure 4. Cell proliferation in (A) MDA-MB-231 and (B) MCF-7 cells. BrdU test after 24 h; (Eth = Dissolved in 70% ethanol, H_2O = dissolved in water), mixture (EGCG + quercetin). Effect of quercetin and EGCG at different concentrations and of estradiol and tamoxifen as controls on the cell viability relative to the negative control (NC) (100%). The mean values with positive standard deviations are represented. *P<0.05.

Oxidative stress. The oxidative stress test was used to evaluate the metabolism of the cells. As an indicator of oxidative stress, an increase in the H_2O_2 concentration was measured in the cell culture.

It was found that in all extract suspensions, the concentration of H_2O_2 increased compared with that in the negative control. GT extracts dissolved in ethanol resulted in the strongest increase in H_2O_2 (up to 1.7 times the values observed in the control). EGCG also showed a factor of 1.5 increase in the H_2O_2 concentration (data not shown).

Similar to MDA-MB-231 cells, the GT extracts dissolved in ethanol and EGCG extract showed the highest H_2O_2 concentrations in MCF-7 cells compared with those in the negative control with a maximum 1.5-fold increase.

Discussion

This study confirmed that EGCG and quercetin are contained in GT and that the single substances, as well as GT itself, have an anticarcinogenic effect on both ER/PR receptor-positive and -negative breast cancer cells, suggesting estrogen receptor-independent pathways.

EGCG is the most abundant anticarcinogenic catechin in GT (4). In the German breast cancer guideline of 2012, EGCG has been previously described as a complementary drug used by patients that should not be applied outside of clinical trials (16). In the ATP luminescence test, a correlative (|r|=0.97) concentration-dependent inhibition of cell viability was observed in both cell lines. At the highest concentration tested, the effect of EGCG corresponded with that of tamoxifen. Wang *et al* performed the same ATP assay with MCF-7 cells and EGCG, testing EGCG concentrations between 4.5 and 54 μg/ml. These researchers' results showed an inhibition of cell viability even at low concentrations after 24 h. In the MTT assay, Wang *et al* reported viability values after 48 h that are comparable to those of our study (18). In MDA-MB-231 cells, Thangapazham *et al* (17) described lower viability values in the MTT assay with EGCG at different

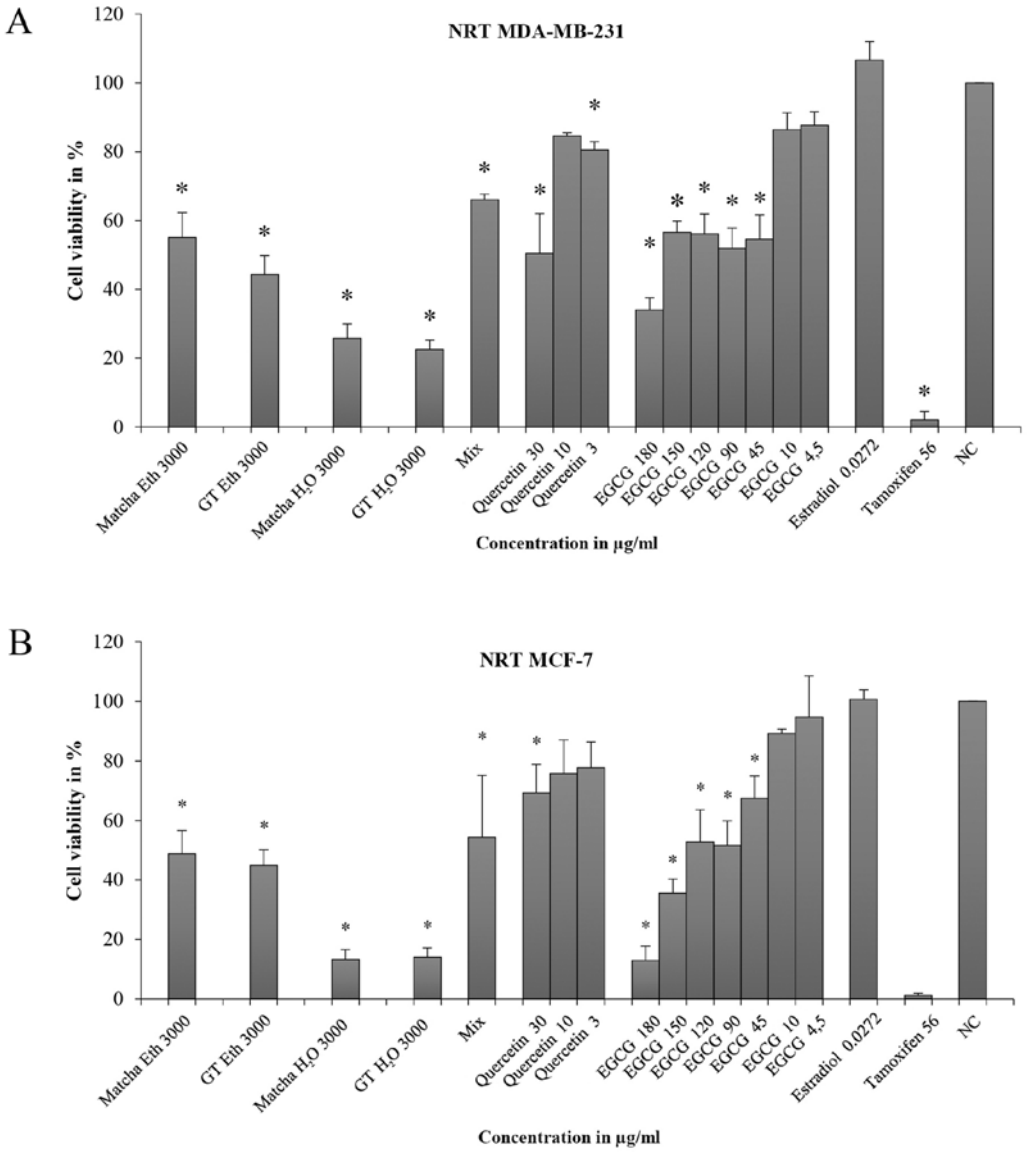

Figure 5. Cell viability in (A) MDA-MB-231 and (B) MCF-7 cells. Neutral Red Test; (Eth = dissolved in 70% ethanol, H_2O = dissolved in water), mixture (EGCG + quercetin). Effect of quercetin and EGCG at different concentrations and of estradiol and tamoxifen as controls on the viability relative to the negative control (NC) (100%). The mean values with positive standard deviations are represented. *P<0.05.

concentrations after 48 h. The ED_{50} was reached at EGCG 50 μg/ml; however, in this study, the ED_{50} was not achieved by any of the tested concentrations. In the studies of both Wang *et al* and Thangapazham *et al*, the measurements were also carried out after 24, 48 and 72 h, and even 96 h, as reported by Thangapazham *et al*. All of the results showed that the cell viability was decreased further over time. An explanation for the difference in viability values, despite identical test methods and identical EGCG concentrations, could be the pH value. As early as 1997, Zhu *et al* reported that catechins are considerably more stable at acidic pH values than at alkaline pH values (19). Our pH measurements always showed alkaline pH values, suggesting instability of the EGCG molecules. In the investigations of Wang *et al* and Thangapazham *et al*, no information was available on the pH values of the cell cultures. Therefore, it is possible that they worked at lower pH values, leading to higher stability of the catechins and increased inhibition of cellular viability.

Not every EGCG concentration in the BrdU test in MCF-7 cells reached the ED_{50}. However, in MDA-MB-231 cells, the mixture of EGCG and quercetin exhibited a stronger effect than the two single substances alone. An explanation could be the influence of the two substances on the PI3K/Akt/mTOR signaling pathway and thus on endothelial growth factor (VEGF). Van Aller *et al* (20) demonstrated that EGCG has an inhibitory effect on this signaling pathway in MDA-MB-231 cells. Bruning confirmed the inhibitory effect of quercetin on mTOR (21). Gu *et al* observed that the oral administration of EGCG in mice resulted in a VEGF-mediated reduction of tumor volume compared with the control group (22). Wang *et al* demonstrated in 2014, using a xenograft prostate tumor mouse model, that the combination of EGCG and quercetin resulted in greater bioavailability and lower methylation of the catechins. Tumor size could be reduced compared with that in the control groups. They also found that the quercetin and EGCG combination led to a significant reduction in phosphorylated AKT

(pAKT) (23). Scandlyn *et al* combined EGCG with tamoxifen, provoking a reduction of mTOR by 78% in receptor-negative tumors *in vivo* (24).

Our results showed no significant differences between the effects of EGCG on estrogen receptor-negative MDA-MB-231 and ER-positive MCF-7 cells; therefore, it can be assumed that the effect is not, or at least not exclusively, generated via the estrogen receptor. Tachibana *et al* (26) and Umeda *et al* (25) found that EGCG exerts its anticarcinogenic effect on its binding behavior with the surface the 67-kDa laminin receptor (67-LR). The 67-LR is important in the metastasis of tumor cells (27). Mittal *et al* showed that EGCG inhibits telomerase in MCF-7 cells, which is increased in more than 90% of all cases in tumors (28). Roy *et al* (29) described increased expression of p53 and the proapoptotic protein Bax, as well as reduced expression of the anti-apoptotic protein Bcl-2 in estrogen receptor-negative breast cancer cells under EGCG influence. Moreover, although MDA-MB-231 cells are triple negative, these cells express ERβ. Therefore, tamoxifen treatment is possible (30).

In the tests performed using GT and MT extracts, a reduction in cellular viability, vitality, and proliferation was observed (Fig. 2A and B). It is assumed that EGCG is responsible for most of the anticancer effects of GT (4,31). As demonstrated in our results, Wang *et al* observed significantly higher viability values in the MTT assay than the ATP measurements (18). An explanation could be offered by the studies of Bruggisser *et al* (32) and Peng *et al* (48), who observed that antioxidants and flavonoids result in a reduced reaction of MTT to the dye formazan, even without the presence of living cells. The amount of formazan formed is measured photometrically, and false-high results are measured.

The flavonoid quercetin was detected by HPLC in GT. In almost all tests performed, inhibition of cell viability was demonstrated, except for the MCF-7 cells in the MTT assay, in which all results were above 100% cell viability but without a statistical significance. In a study by Scambia *et al*, quercetin inhibited the growth of MCF-7, as well as that of MDA-MB-231 cells, in a dose-dependent manner by binding to the estrogen-binding domain type II (EBS II) (33). Lee *et al* described that the induction of apoptosis of MCF-7 cells is caused by the formation of free oxygen radicals that activate the pro-apoptotic AMPKα1/ASK1/p38 signaling pathways (34). Duo *et al* recognized the apoptotic effect of quercetin in the upregulation of Bax protein and reduction of Bcl-2 protein (35). Similar to EGCG, quercetin also has an inhibitory effect on the mTOR signal cascade (36).

As already mentioned, EGCG is a very sensitive molecule. It is assumed that EGCG changes its structure under cell culture conditions by auto-oxidation and dimerization. Sang *et al* observed increased instability of the EGCG molecule in normal tap water, attributable to its iron constituents (37). Other factors that influence the molecular stability are pH, temperature and the concentration of EGCG (20,37). Sang *et al* demonstrated that 20 μmol/l of EGCG in RPMI-1640 medium (named after the Roswell Park Memorial Institute), similar to the DMEM used in our work, lost integrity after 1 h at 37˚C. At high concentrations, EGCG tends to change the stereoconformation and become GCG (gallocatechingallate) and the molecules' half-life is prolonged. At lower concentrations, dimer formation occurs, resulting in molecular instability. Inconsistent amounts of time passed between the preparation and measurement may contribute to the heterogeneous results in the literature on the amount of EGCG in a cup of tea, presuming that some of the EGCG may have already decayed after the preparation of the extracts at the time of the determination of the quantity (37).

Importantly, this study was performed *in vitro*; therefore, the bioavailability of EGCG in humans was not considered. Yang *et al* found in a patient study (n=18) that the consumption of 3 g of decaffeinated GT (EGCG content: 73 mg/g) resulted in a plasma concentration of no more than 326 ng/ml. However, an increase from 1.5 to 3 g resulted in a triple EGCG plasma concentration; a further increase at a dose of 4.5 g could not be observed. Thus, it appears that a saturation of EGCG uptake is achieved around 3 g (38). Chow *et al* tested the bioavailability of EGCG by administering EGCG capsules. They measured the plasma levels of 439 ng/ml after administering 800 mg of EGCG or 388 ng/ml after the administering 800 mg of uncontaminated EGCG (polyphenol E) (39). These results show that the bioavailability of EGCG in plasma is very low.

One way to increase EGCG bioavailability is described by Landis-Piwowar *et al*, who demonstrated *in vitro* as well as in animal experiments *in vivo* that a prodrug of EGCG, in which the hydroxyl groups were protected by peracetic acid groups before methylation, leads to greater bioavailability and results in higher concentrations in breast cancer cells (40). Moreover, in 2007, they found that the methylation of EGCG by catechol *O*-methyltransferase (COMT) leads to decreased anticarcinogenic activity of the catechins (41). This observation was also consistent with the work of Wu *et al* who demonstrated that predominantly, carriers of the COMT-L allele, a low-activity form of COMT, benefited from the anti-carcinogenic effects of the catechins; however, in carriers of the highly active COMT-H allele, there was no difference in breast cancer risk between tea consumers and non-consumers (42). Landis-Piwowars *et al* demonstrated that, in MDA-MB-231 cells expressing the COMT-H form, the effect of catechins can be increased using COMT inhibitors (43).

In conclusion, based on our results and the findings reported in the literature, the anti-carcinogenic activity by GT and its components EGCG and quercetin can be assumed.

A problem in the therapeutic setting of EGCG is the low oral bioavailability. In animal studies (e.g., in athymic mice), the possibility of intravenous EGCG administration to avoid the first-pass effect could be tested (44). Another approach would be to administer an EGCG prodrug, as described by Landis-Piwowar *et al*, leading to better bioavailability (40).

However, more studies are crucial to determine the potential toxicity of EGCG at higher doses, as hepatotoxic effects at high concentrations are described. The drug interactions of EGCG and associated risks, e.g., effects of the pharmacokinetics of concomitant medication by influencing enzymes such as the cytochrome P450 system, need further evaluation (45). It is also worth including Her-2 positive cell lines in further studies. But for this study, we focused on ERα/ERβ-positive cells.

EGCG and tamoxifen could represent a promising combination in breast cancer therapy as synergistic anti-proliferative and cytotoxic effects in both MCF-7 and MDA-MB-231 cells

were observed (46,47). Scandlyn *et al* observed an anti-proliferative effect of EGCG in combination with low tamoxifen doses (75 μg/kg) in animals with triple-negative breast cancer (24). To better understand this effect, active compounds of the two substances and pathways associated should be investigated more intensively. Therefore, studies regarding the influence on apoptosis and neovascularization should be considered. To understand the mode of action of EGCG, it would be interesting to further investigate the role of the 67-kDa laminin receptor (25,27).

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

LS and PM performed the experiments. SH added additional experiments. DUR conceived and designed the study. JGK, HH, TV, TPB, SM and UJ were also involved in the conception of the study and revised it critically. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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