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Epigallocatechin gallate & curcumin prevent transforming growth factor beta 1-induced epithelial to mesenchymal transition in ARPE-19 cells

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***Background & objectives*: Proliferative vitreoretinopathy (PVR) is characterized by the presence of epiretinal membrane (ERM), which exerts traction and detaches the retina. Epithelial to mesenchymal transition (EMT) of the retinal pigment epithelial (RPE) cells underlies ERM formation. Adjuvant therapies aimed at preventing recurrence of PVR after surgery mostly failed in clinical trials. This study was aimed to evaluate the anti-EMT properties of bio-active compounds epigallocatechin gallate (EGCG), curcumin and lycopene as inhibitors of EMT induced by transforming growth factor beta 1 (TGF-β1) in cultured ARPE-19 cells.**

***Methods*: ARPE-19 cells were treated with TGF-β1 alone or co-treated with EGCG (1-50 µM), lycopene (1-10 µM) and curcumin (1-10 µM). The mRNA and protein expression of EMT markers, alpha-smooth muscle actin, vimentin, zonula occludens-1 and matrix metalloproteinase-2 (MMP-2), were assessed by reverse transcription polymerase chain reaction/quantitative polymerase chain reaction and immunofluorescence/enzyme linked immunosorbent assay. Activity of MMP-2 was assessed by zymography. Functional implications of EMT were assessed by proliferation assay (MTT assay) and migration assay (scratch assay). Western-blot for phosphorylated Smad-3 and total Smad-3 was done to delineate the mechanism.**

***Results*: EGCG and curcumin at 10 µM concentration reversed EMT, inhibited proliferation and migration through Smad-3 phosphorylation, when induced by TGF-β1 in ARPE-19 cells. Lycopene did not prevent EMT in ARPE-19 cells.**

***Interpretation & conclusions*: EGCG and curcumin are potent in preventing EMT induced by TGF-β1 in ARPE-19 cells and therefore, proposed as potential molecules for further pre-clinical evaluation in PVR management.**

Key words Curcumin - epigallocatechin gallate - epithelial to mesenchymal transition - lycopene - proliferative vitreoretinopathy - Smad-3 - transforming growth factor beta 1

Proliferative vitreoretinopathy (PVR) is a wound healing response elicited by retinal detachment (RD) that ends up in the formation of fibrotic tissues. Depending on the location of such fibrotic tissue formation, epithelial to mesenchymal transition (EMT) is called as epi-retinal membrane (ERM) - on the vitreous

surface of retina; sub-retinal membrane - between the retinal pigment epithelial (RPE) and neurosensory retina; posterior hyaloids membrane - on the posterior surface of vitreous. These membranes are collectively called as peri-retinal membrane[1,2]. Membranes formed in PVR consist of predominantly RPE cells, apart from astrocytes and Müller cells. Retinal breaks or trauma disrupts the blood-retinal barrier, resulting in the discharge of RPE cells into the vitreous[1,2]. Growth factors in the vitreous trans-differentiate the RPE cells into fibroblast-like cells as seen in ERM[3]. Both *in vivo* and *in vitro* studies have shown that this trans-differentiation of RPE cells into fibroblast is due to EMT and that transforming growth factor beta 1 (TGF-β1) is a well-known inducer of EMT in RPE cells[4,5]. Thus, EMT plays a key role in the pathology of PVR. Hence, inhibition of EMT can be pivotal in the adjuvant therapy of PVR. Inflammation, proliferation or inhibition of specific growth factors were the target of adjuvant agents tested so far, but the clinical outcome is not satisfactory[1]. Better adjuvant to prevent surgical failure is required. Hence, this study was undertaken to screen (-) epigallocatechin gallate (EGCG), an important constituent of *Camellia sinensis* (green tea), curcumin, a bioactive compound from *Curcuma longa* (turmeric), and lycopene, from *Solanum lycopersicum* (tomato), for their anti-EMT activity if any, in RPE cells. These compounds were chosen considering their chemo-preventive and anti-invasive properties against various tumours[6-9]. Since invasiveness of a tumour is dependent on EMT, the study was also aimed to see if any of these compounds could inhibit EMT in ARPE-19 cells and their mechanisms of action.

Material & Methods

Cell culture and treatment: Adult human RPE cells ARPE-19 (ATCC-CRL-2302) purchased from ATCC (Virginia, USA) were cultured using DMEM-F12 (Sigma, USA) supplemented with 10 per cent foetal bovine serum (FBS, Gibco, Life Technologies, USA), 14.2 mM sodium bicarbonate and 1X solution of antibiotic-antimycotic (Gibco, Life Technologies).

Preparation of epigallocatechin gallate (EGCG), curcumin and lycopene: Lycopene and curcumin (Sigma, USA) were dissolved in tetra hydrofuran (THF) containing 0.1 per cent butyl hydroxyl toluene and dimethyl sulphoxide (DMSO), to obtain a stock concentration of 1.2 and 13.5 mM, respectively, then serially diluted to 1-10 µM concentrations. EGCG being hydrophilic was dissolved in DMEM-F12 medium+1 per cent FBS to the stock concentration of 7.5 mM and diluted to concentrations of 1, 10 and 50 µM.

Treatment of ARPE-19 cells: ARPE-19 (4×10^4) cells were seeded on a 12-well plate and allowed to reach 80 per cent confluency. Cells were serum starved overnight for synchronization using DMEM-F12+1 per cent FBS; after serum starvation, the cells were treated with TGF-β1 (5 ng/ml) and co-treated with EGCG (1, 10 and 50 µM), lycopene (1-10 µM) or curcumin (1-10 µM). DMSO (2%) and THF (0.8%) were used as vehicle controls. Cells without any treatment served as control. EMT markers were assessed by reverse transcription polymerase chain reaction (RT-PCR), quantitative polymerase chain reaction, immunofluorescence (IF) and enzyme linked immunosorbent assay (ELISA). Proliferation assay and scratch assay were used to assess the effect of these compounds on TGF-β1-induced cell proliferation and migration.

Cytotoxicity assay: Trypan blue dye exclusion assay[10] was used to assess the cytotoxic effects of EGCG, curcumin and lycopene on 80 per cent confluent ARPE-19 cells. After serum starvation, EGCG (1, 10 and 50 µM), lycopene (1-10 µM) or curcumin (1-10 µM) was added and incubated for 48 h. At the end of incubation, the cells were trypsinized and suspended in DMEM-F12. 0.4 ml of 0.4 per cent (w/v) trypan blue was added to 0.4 ml of cell suspension and incubated at room temperature for two minutes, before counting cells using haemocytometer (Rohem Industries, Maharashtra, India). Non-viable cells take up the stain and appear blue, whereas the viable cells remain unstained. Total percentage of viable cells was calculated using the formula:

Viability (%) = Total number of viable cells/Total number of cells counted

Gelatin zymography: After 36 h of treatment under the conditions mentioned above, the cell-free conditioned medium was collected and the total protein concentration was determined by Bradford's reagent (Thermo Scientific, USA); 35 µg of the total protein was used for gelatin zymography as reported previously[11]. Developed zymograms were scanned using Bio-Rad GS800 Calibrated Densitometer (Bio-Rad, USA) and densitometry analysis was carried out using ImageJ software (NIH, USA).

Matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor-A (VEGF-A) estimation

by ELISA: Matrix metalloprotease-2 (MMP-2) and vascular endothelial growth factor-A (VEGF-A) levels were measured using R&D Quantikine ELISA Kit (R&D Systems, USA). Briefly, cell-free conditioned medium was collected after 36 h of treatment. Fifty microlitres of conditioned medium was added to appropriate antibody-coated ELISA plates and incubated at room temperature. It is followed by washing; addition of appropriate horseradish peroxidase-conjugated secondary antibody and colour development using tetramethylbenzidine (TMB) substrate as per the manufacturer's instruction. Colour developed was read spectrometrically in a multi-well plate reader (Spectromax M2E, Molecular Devices, USA).

RNA extraction and RT-PCR: RT-PCR was used to evaluate the effect of EGCG, lycopene and curcumin on mRNA expression levels of EMT markers under treatment conditions. Total RNA was extracted using 500 µl of TRIzol reagent (Life Technologies, USA) per well of 12-well plate following manufacturer's instructions. One microgram of total RNA was converted to cDNA using iScript cDNA Synthesis Kit (Bio-Rad, USA), and RT-PCR was done for EMT markers [alpha smooth muscle actin (*α-SMA*), vimentin, zonula occludens-1 (*ZO-1*), *MMP-2*] and housekeeping gene (*18S rRNA*) using PCR mastermix (New England Biolabs, USA) following the manufacturer's instruction. Table gives the list of primers used. PCR products were resolved on a 2 per cent agarose gel and documented using FluorChemFC3 system (ProteinSimple, USA). NIH ImageJ software was used for densitometry analyses. Quantitative real-time PCR using SYBR Green chemistry was done to validate the results of RT-PCR using Roche LightCycler® 96 machine (Roche Diagnostics, Switzerland) using the primers (Table). Comparative $2^{-\Delta\Delta Ct(treated-untreated)}$ method was used to calculate the fold change[12].

Immunocytochemistry: ARPE-19 cells were grown to 80 per cent confluence in four-well chamber slides (Nalge Nunc, USA). After treatment as mentioned above, immunocytochemistry was done to see the expression of α-SMA, vimentin and ZO-1, using primary antibodies against α-SMA (1:100) (Sigma, USA), vimentin (1:100) or ZO-1 (1:75) (Abcam, UK) and appropriate Alexa-488 conjugates secondary antibody (Invitrogen, USA) and the nucleus was stained with DAPI. Five random images were taken from a single chamber in the slide using

Table. List of primers used for reverse transcriptase polymerase chain reaction and quantitative polymerase chain reaction

Gene	Forward primer	Reverse primer	Reference	Size (base pair)
MMP-2 (RT-PCR)	5'-GCAGATGCCTGGAATGCCAT-3'	5'-AGGGTTCTGTGAGCCACAGA-3'	44	523
α-SMA (RT-PCR)	5'-ATCACCATCGGAAATGAACG-3	5'-CTGGAAGGTGGACAGAGAGG-3'	6	318
Vimentin (RT-PCR)	5'-GAGAACTTTGCCGTTGAAGC-3'	5'-CGTGATGCTGAGAAGTTTCG-3'	6	343
ZO-1 (RT-PCR)	5'-CCAGAATCTCGGAAAAGTGC-3'	5'-ACCGTGTAATGGCAGACTCC-3'	6	397
18S rRNA (RT-PCR/qPCR)	5'-GTGGAGCGATTTGTCTGGTT-3'	5'-GGACATCTAAGGGCATCACAGA-3'	45	165
MMP-2 (qPCR)	5'-CAGGAGGAGAAGGCTGTGTT-3'	5'-TTAAAGGCGGCATCCACTCG-3'	12	137
α-SMA (qPCR)	5'-GGCTGTTTTCCCATCCATTGT-3'	5'-TCTTTTGCTCTGTGCTTCGT-3'	12	103
Vimentin (qPCR)	5'-TTGCAGGAGGAGATGCTTCA-3'	5'-TTGCGTTCAAGGTCAAGACG-3'	12	104
ZO-1 (qPCR)	5'-AGTAAGTCGTCCTGATCCTGAACC-3'	5'-TCCTTCTGTTAACCACACCACTCC-3'	12	107

RT-PCR, reverse transcriptase polymerase chain reaction; qPCR, quantitative polymerase chain reaction; *α-SMA*, alpha smooth muscle actin; MMP-2, matrix metalloprotease-2; *ZO-1*, zonula occludens-1

Carl-Zeiss Epifluorescence Microscope (Carl Zeiss AG, Germany) for analysis.

Scratch assay for migration: A scratch was made in confluent culture of ARPE-19 cells with a sterile 200 µl pipette tip as described previously[11]. Floating cells were removed by washing with DMEM-F12 medium. Cells were treated as indicated and photographed using phase contrast microscope (EVOS-XL-core, American Microscopy group, Life Technologies, USA) at the time of scratch (zero hour) and after 36 hours.

Cell proliferation assay: Cell proliferation assay was done as described in MTT Cell Proliferation Kit (ATCC, USA) with modifications. Briefly, ARPE-19 cells (2.5×10^3) were seeded per well of 96-well plates and allowed to attach overnight. Cells were treated with TGF-β1 alone or co-treated with EGCG, curcumin and lycopene as indicated for 48 h. At the end of incubation, 180 µl of fresh DMEM-F12+1 per cent FBS and 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [final concentration (1mg/ml)] were added. After incubation for 3.5 h in the CO_2 incubator, the medium was carefully aspirated and formazon crystals formed were dissolved in 100 µl DMSO. Absorbance of formazon crystal is related to the cell number and hence is a measure of cell proliferation. The absorbance of formazon crystal was measured spectrophotometrically at 570 nm with a reference wavelength of 650 nm using a multi-well reader (Spectromax M2E, Molecular Devices, USA).

Western blot for Smad-3 phosphorylation: ARPE-19 cells were treated as indicated and the total protein was extracted using M-PER Protein Extraction Reagent (Fisher Scientific, USA) and the concentration was estimated by BCA method (Thermo Scientific, USA). Twenty five micrograms of total protein was resolved on a 10 per cent polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham™ Hybond, Sigma, USA) by semi-dry transfer. Membrane was probed overnight with rabbit phosphorylated-Smad-3 [p-Smad-3 (1:5000)], total Smad-3 [Smad-3(1:4000)] or mouse β-actin antibody (1:8000) (Santa Cruz, USA), followed by incubation with appropriate anti-rabbit and anti-mouse secondary antibodies. Blots were developed using Clarity™ Western ECL Substrate (Bio-Rad, USA) and imaged using Fluorchem-Fc3 (ProteinSimple, USA). Intensity of bands was assessed using ImageJ software. p-Smad-3/Smad-3 ratio normalized to β-actin was used to measure the phosphorylation status of Smad-3.

Statistical analysis: Data were expressed as mean±standard deviation of three independent experiments performed in triplicates. ANOVA with Bonferroni correction was done to compare the difference among the groups.

Results

Cytotoxicity assessment of EGCG, curcumin and lycopene in ARPE-19 cells: Trypan blue exclusion assay showed that EGCG up to 50 µM and curcumin and lycopene up to 10 µM were not cytotoxic to ARPE-19 cells (Fig. 1). These concentrations were further screened for anti-EMT activity.

Effect of EGCG, curcumin and lycopene on EMT markers: The concentration range from 1 to 10 µM of curcumin and lycopene was used as concentration of lycopene >10 µM started to precipitate in the medium, while curcumin at 15 µM concentration induced cell death (data not shown). Co-treatment with TGF-β1 and 1-10 µM of either curcumin or lycopene showed that 10 µM of curcumin reversed the mRNA expression of EMT markers, namely *α-SMA*, vimentin and *MMP-2* that were augmented by TGF-β1. However, lycopene did not show any effect on *α-SMA*, vimentin or *MMP-2*, but it rescued the *ZO-1* mRNA levels. Comparison of anti-EMT activity of the nutraceuticals showed that EGCG (10 and 50 µM) and curcumin (10 µM) reduced

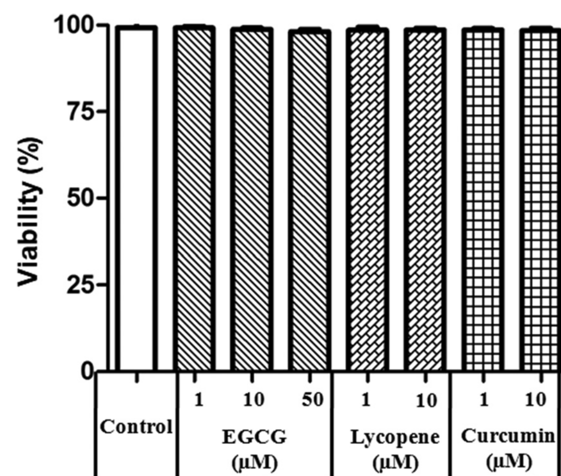

Fig. 1. Cell viability assay: Trypan blue exclusion assay was done after treating ARPE-19 cells with various concentrations of epigallocatechin gallate (EGCG, 1, 10 and 50 µM); lycopene (1 and 10 µM) and curcumin (1 and 10 µM) for 48 h. Data represent mean±standard deviation of three independent experiments.

the mRNA expressions of EMT markers, *α-SMA*, vimentin and *MMP-2* significantly ($P<0.001$). RT-PCR showed that mRNA expression of EMT marker *α-SMA* (*α-SMA/18S rRNA*) was significantly increased by TGF-β1 (1.38±0.13, $P<0.001$) compared to control (0.61±0.02) and it was reduced by 10 μM EGCG (0.52±0.08, $P<0.001$), 50 μM EGCG (0.31±0.08, $P<0.001$) and 10 μM curcumin (0.40±0.09, $P<0.001$). mRNA expression of vimentin was significantly increased by TGF-β1 (1.42±0.06, $P<0.001$) compared to control and it was reduced by 10 μM EGCG (0.64±0.08, $P<0.001$), 50 μM EGCG (0.46±0.05, $P<0.001$) and 10 μM curcumin (0.65±0.007, $P<0.001$). *MMP-2* mRNA expression (*MMP-2/18S rRNA*) was significantly increased by TGF-β1 (1.4±0.18, $P<0.001$) compared to control and it was reduced by 10 μM

Fig. 2. Effect of EGCG, curcumin and lycopene on mRNA expression of epithelial to mesenchymal transition markers reverse transcription polymerase chain reaction: ARPE-19 cells were treated with TGF-β1 alone or co-treated with EGCG (1, 10 and 50 μM), curcumin (1 and 10 μM) and lycopene (1 and 10 μM) for 36 h. (**A**) Representative gel picture showing the effect of compounds at mRNA levels of *MMP-2*, vimentin, *α-SMA* and *ZO-1*. (**B**) Bar chart representing densitometry analysis of semi-quantitative reverse transcription polymerase chain reaction. Data represent mean±standard deviation of three independent experiments in triplicates. $^{***}P<0.001$ compared to control; P $^{\#}<0.05$, $^{\#\#}<0.001$, $^{\#\#\#}<0.001$ compared to TGF-β1. TGF-β1, transforming growth factor beta 1; ZO-1, zonula occludens-1; MMP-2, matrix metalloproteinase-2; α-SMA, alpha smooth muscle actin.

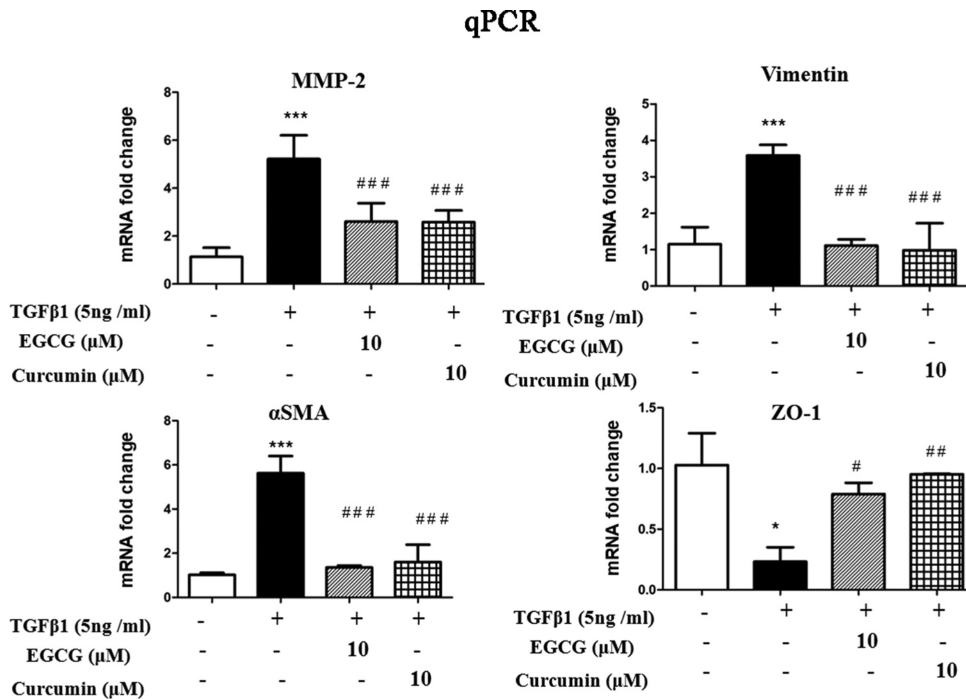

Fig. 3. Effect of EGCG, curcumin on mRNA expression of epithelial to mesenchymal transition markers-quantitative polymerase chain reaction: ARPE-19 cells were treated with transforming growth factor beta 1 alone or co-treated with EGCG (10 μM), curcumin (10 μM) for 36 h; mRNA expression of *MMP-2*, vimentin, *α-SMA* and *ZO-1* were assessed by quantitative real-time polymerase chain reaction. Data represent mean±standard deviation of three independent experiments in triplicates. $P^{*}<0.05$, $^{***}<0.001$ compared to control; $P^{\#}<0.05$, $^{\#\#}<0.001$, $^{\#\#\#}<0.001$ compared to TGF-β1.

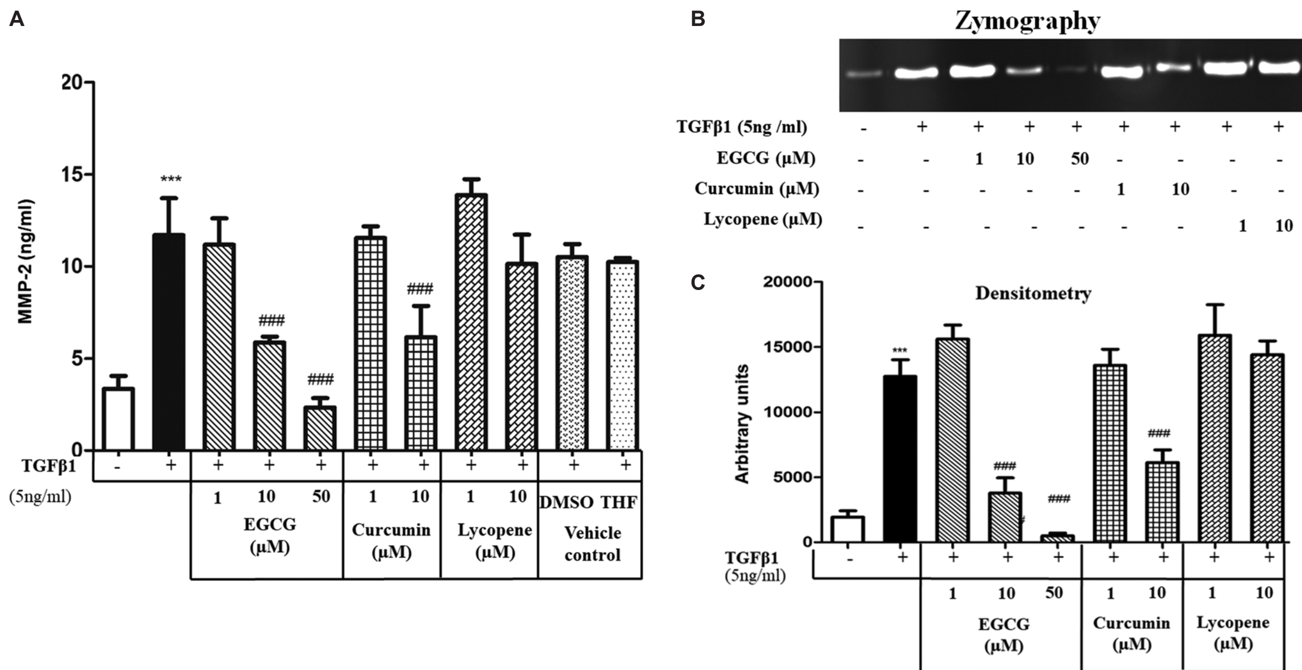

Fig. 4. Effect of EGCG, curcumin, lycopene on MMP-2: Cell free conditioned medium, from ARPE-19 cells treated with TGF-β1 alone or co-treated with EGCG (1, 10, 50 μM), curcumin (1 and 10 μM) and lycopene (1 and 10 μM) for 36 h was used. (**A**) Estimation of MMP-2 levels in condition media using ELISA. (**B**) Zymography showing MMP-2 activity in treated conditions. (**C**) Bar chart representing densitometry analysis of MMP-2 activity. Data represent mean±standard deviation of three independent experiments. $^{***}P<0.001$: Comparison between control and TGF-β1; $^{\#\#\#}P<0.001$: Comparison between TGF-β1 and corresponding treatment. ELISA, enzyme linked immunosorbent assay.

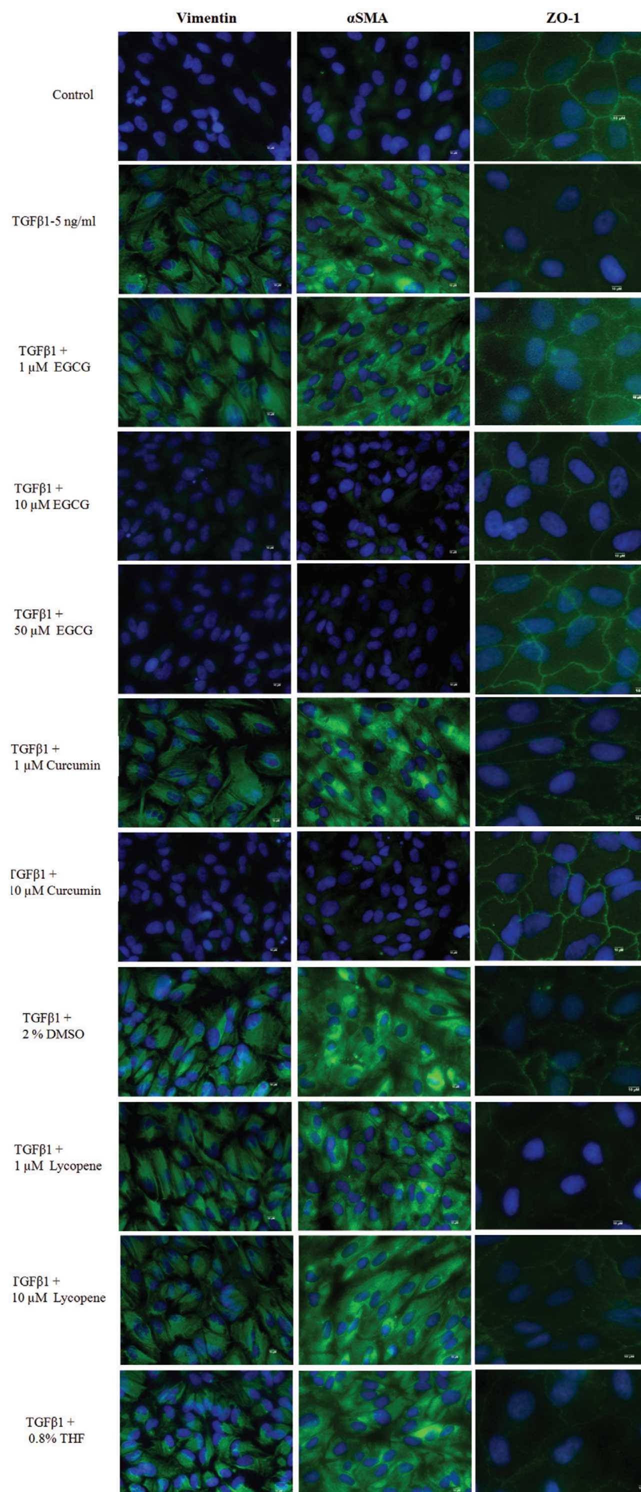

Fig. 5. Effect of EGCG, curcumin and lycopene on expression of vimentin, α-SMA and ZO-1: ARPE-19 cells treated with TGF-β1 or co-treated with indicated concentration of EGCG, curcumin or lycopene for 48 h and stained for α-SMA, vimentin and ZO-1 (green fluorescence) and nucleus was stained with DAPI (blue fluorescence). All the images were taken using Carl-Zeiss fluorescence microscope (×40).

EGCG (0.67±0.1, $P<0.001$), 50 µM EGCG (0.54±0.07, $P<0.001$) and 10 µM curcumin (0.70±0.05, $P<0.001$). There was no significant difference between EGCG and curcumin in reducing the mRNA expression of *α-SMA*, vimentin and *MMP-2* in the studied concentrations. Compared to control (1.18±0.09), *ZO-1* mRNA levels were downregulated by TGF-β1 (0.54±0.03, $P<0.001$), were reversed by 10 µM EGCG (0.80±0.10, $P<0.001$), 50 µM EGCG (0.84±0.12, $P<0.001$), 1 µM lycopene (0.82±0.05, $P=0.001$), 10 µM lycopene (0.76±0.08, $P<0.001$), 1 µM curcumin (0.77±0.18, $P<0.001$) and 10 µM curcumin (0.77±0.11, $P<0.001$) (Fig. 2). Quantitative real-time PCR was done to validate the effect of EGCG and curcumin on the expression of EMT markers. It showed that TGF-β1 induced the expression of *MMP-2* (5.2±0.98-fold, $P<0.001$), vimentin (3.6±0.3-fold, $P<0.001$), *α-SMA* (5.6±0.7-fold, $P<0.001$) and downregulated the expression of *ZO-1* (0.23±0.11-fold, $P=0.02$), which was significantly reversed by 10 µM EGCG (*MMP-2* 2.6±0.7-fold, $P=0.0057$; vimentin 1.10±0.17-fold, $P<0.001$; *α-SMA*: 1.3±0.08-fold, $P<0.001$; *ZO-1* 0.78±0.09-fold, $P=0.01$) and 10 µM curcumin (*MMP-2* 2.58±0.48-fold, $P<0.0001$; vimentin 0.98±0.74-fold, $P<0.001$; *α-SMA*: 1.6±0.78-fold, $P<0.001$; *ZO-1* 0.98±0.03-fold, $P=0.007$) (Fig. 3).

The effect of EGCG, curcumin and lycopene on protein expression and activity of MMP-2 was determined by ELISA and zymography. Treatment of TGF-β1 significantly induced the expression of MMP-2 (11.7±2.0 ng/ml, $P\leq0.001$) compared to control (3.35±0.7 ng/ml). MMP-2 expression was downregulated by 10 µM EGCG (5.85±0.3 ng/ml, $P=0.001$); 50 µM EGCG (2.34±0.5 ng/ml, $P=0.001$) and 10 µM curcumin (6.1±1.7 ng/ml, $P\leq0.001$) (Fig. 4A). MMP-2 activity by zymography also showed that EGCG (10 and 50 µM) and curcumin (10 µM) reduced the activity of MMP-2, which was upregulated by TGF-β1 (Fig. 4B & C).

The protein expression of α-SMA, vimentin and ZO-1, on treatment with the nutraceuticals, was assessed by IF (Fig. 5). TGF-β1 induced the expression of mesenchymal markers, α-SMA and vimentin and decreased the expression of ZO-1 compared to control. EGCG (10 and 50 µM) and curcumin (10 µM) reversed the expression of EMT markers. Lycopene and the vehicle controls did not show any effect on EMT markers.

ARPE-19 cells treated with TGF-β1 showed elongated fibroblastic morphology as observed with

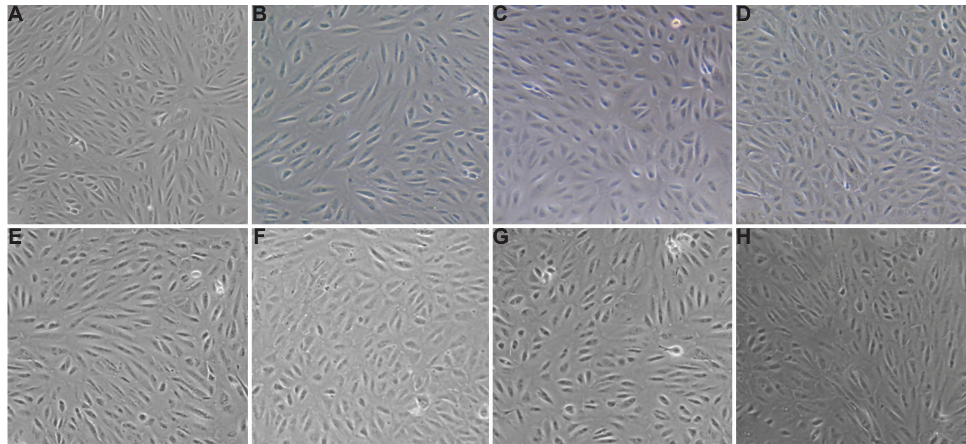

Fig. 6. Morphology of ARPE-19 cells in treated conditions. Phase contrast microscope images of ARPE-19 cells treated with TGF-β1: No treatment (**A**); cell treated with TGF-β1 alone (**B**); co-treated with EGCG 1μM (**C**) and 10 μM (**D**); curcumin 1 μM (**E**) and 10 μM (**F**); lycopene 1μM (**G**) and 10 μM (**H**).

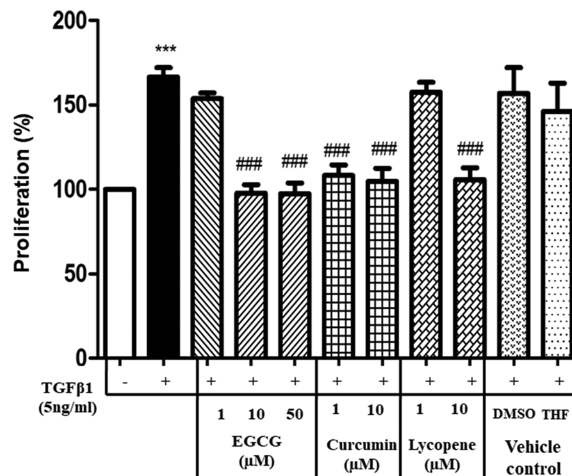

Fig. 7. Effect of EGCG, curcumin and lycopene on proliferation of ARPE-19 cells. MTT assay was done after treating ARPE-19 cells with TGF-β1 and co-treated with EGCG, curcumin or lycopene for 48 h. The bar graph represents per cent proliferation of cells as normalized to control. Data represent mean±standard deviaton of three independent experiments in triplicates. $^{***}P<0.001$ compared to control, $^{###}P<0.001$ compared to TGF-β1. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

phase contrast microscope. The cells treated with EGCG and curcumin showed dose-dependent changes in morphology. EGCG (10 μM) and curcumin (10 μM) reduced the elongated fibroblastic appearance of ARPE-19 cells compared to TGF-β1-treated cells. However, lycopene-treated cells did not show any difference in morphology compared to TGF-β1-treated cells (Fig. 6).

Effect of EGCG, curcumin and lycopene on proliferation of ARPE-19 cells: MTT cell proliferation assay showed that TGF-β1 induced proliferation by 66 per cent ($P<0.001$), which was reverted by EGCG (48% at 10 μM, $P<0.001$; 49% at 50 μM, $P<0.001$) and curcumin (36% at 1 μM, $P<0.001$ and 50% at 10 μM, $P<0.001$) and lycopene (40.06% at 10 μM, $P<0.001$) (Fig. 7).

Scratch assay showed that TGF-β1 induced migration of ARPE-19 cells was reduced by EGCG and curcumin dose dependently. However, lycopene did not show any effect on the migration (Fig. 8).

ELISA for VEGF-A showed that TGF-β1 induced VEGF-A expression (2097±365.2 pg/ml, $P<0.001$) compared to control (660±133.4 pg/ml), which was reduced dose dependently by EGCG (1 μM: 546.8±58.37 pg/ml, $P<0.001$; 10 μM: 235.1±39.2 pg/ml, $P=0.001$; 50 μM: 210.8±19.55 pg/ml, $P<0.001$) and curcumin (1 μM: 632.2±28.17 pg/ml, $P<0.001$; 10 μM: 340.4±21.6 pg/ml, $P<0.001$) (Fig. 9).

The effect of EGCG and curcumin on Smad-3 phosphorylation was studied. TGF-β1-induced Smad-3 phosphorylation (2±0.5-fold, $P<0.05$) was reduced by EGCG (0.86±0.1, $P<0.05$) and curcumin (1.2±0.1-fold, $P<0.05$) but not lycopene (1.94±0.3-fold, $P=0.73$) (Fig. 10).

Discussion

This study findings suggested that EGCG and curcumin inhibited EMT induced by TGF-β1 in ARPE-19 cells. TGF-β1 is shown to be present in the ERM, sub-retinal fluid and vitreous aspirates of PVR subjects[13]. TGF-β1 was generally used to induce EMT in ARPE-19 cells even though both TGF-β1 and β2 induced the expression of α-SMA and MMP-2 and the migration of ARPE-19 cells[4]. Our data also suggested that TGF-β1 induced the expression of EMT markers

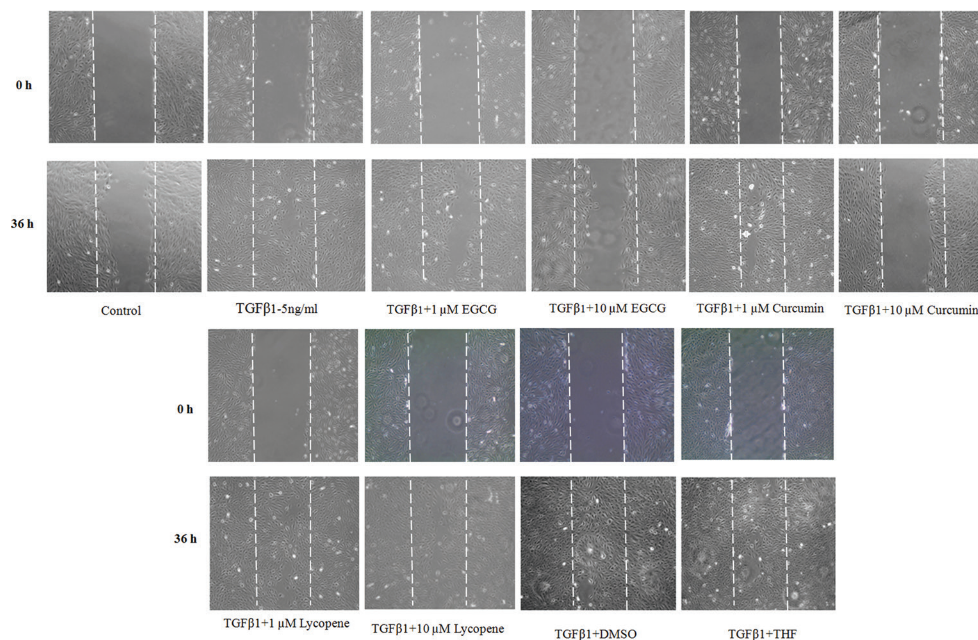

Fig. 8. Effect of EGCG, curcumin and lycopene on migration of ARPE-19 cells. Representative images showing scratch assay on ARPE-19 cells, treated with TGF-β1 alone or co-treated with various concentrations of EGCG, curcumin and lycopene. Cells were imaged immediately after the scratch was made *i.e.* at 0 h and 36 h after the scratch (×4).

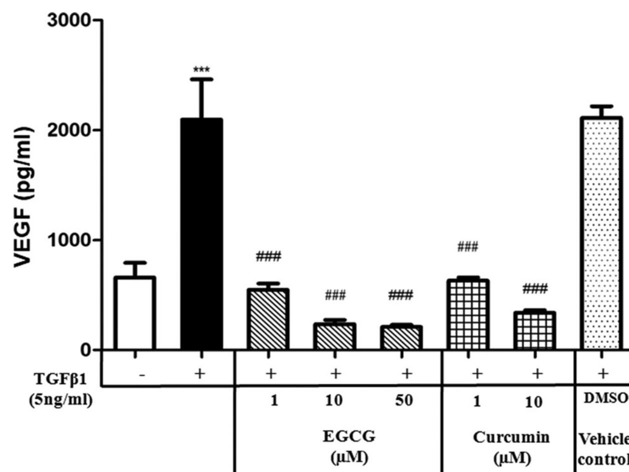

Fig. 9. Effect of EGCG, curcumin on VEGF secretion: ARPE-19 cells treated with TGF-β1 or co-treated with various concentrations of EGCG and curcumin for 48 h. Conditioned media were used to quantify VEGF levels by ELISA. Data represent mean±standard deviation of three independent experiments in triplicates. $^{***}P$<0.001 compared to control, $^{###}P$<0.001 compared to TGF-β1. VEGF, vascular endothelial growth factor.

such as vimentin, α-SMA, MMP-2. Vimentin[3] and α-SMA[13] are abundantly seen in the ERM surgically removed from PVR cases. These proteins are not just EMT markers, these also contribute to the contractility of ERM leading to RD. Increased MMP-2 has been reported in vitreous and subretinal fluid aspirated from PVR cases[14]. MMPs can degrade junctional proteins namely ZO-1 and occludin[15], thereby aiding in the detachment of RPE cells during EMT.

Adjuvant therapy of PVR is aimed at preventing its relapse following retinal re-attachment surgery for RD or PVR. Anti-inflammatory agents, antiproliferative agents and molecules against cytokine signalling tested in clinical trials were found to have inconsistent results[1]. Hence, there is a need for a safe and potent inhibitor for the management of PVR.

EGCG is generally considered safe and does not have acute toxic[16], genotoxic or teratogenic effects[17] as studied in rodents. It has also been reported to be safe in healthy individuals at a concentration of 800 mg/day[18]. Intravitreal injection of EGCG has also been found to be safe upto a dosage of 57 μM[19]. It is also known for its antioxidant activity and maintenance of health status of normal cells and also noted for its antitumour and anti-angiogenic activity. Curcumin has been shown to have antitumour and anti-inflammatory activities[20,21]. Despite concerns about curcumin due to poor bio-availability and solubility[22], curcumin was found to be safe up to 2.2 g[23]. Lycopene is a carotenoid present in significant quantities in tomato. Giovannucci[9] showed that lycopene uptake was inversely proportional to the occurrence of breast, gastric and prostate cancer. Lycopene was also found to be safe in humans with the bio-availability of 30 mg exhibiting antioxidant

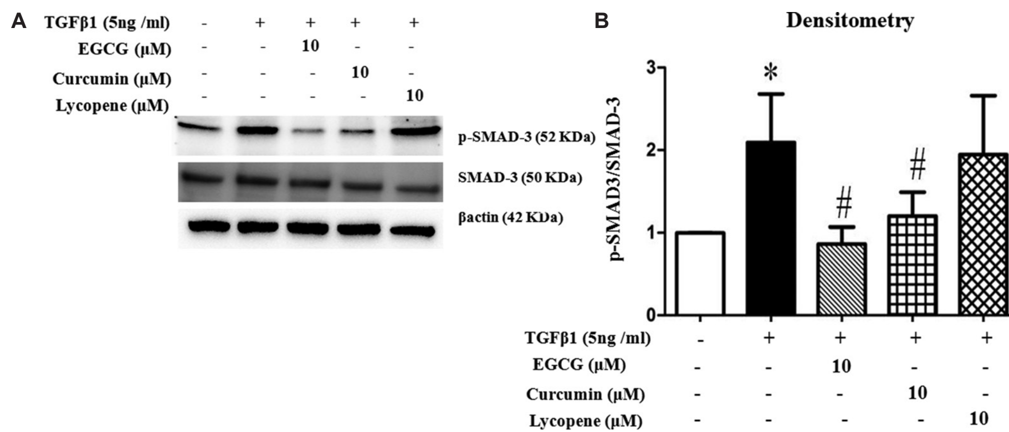

Fig. 10. Effect of EGCG, curcumin and lycopene on Smad-3 phosphorylation: (**A**) EGCG, curcumin and not lycopene reduced the phosphorylation of Smad-3 induced by TGF-β1 in ARPE-19 cells. (**B**) Densitometry is represented as bar graph. Data represent mean±standard deviation of three independent experiments in triplicates. *P<0.05 compared to control, #P<0.05 compared to TGF-β1.

properties[24]. Since these compounds are potentially safe and have shown anticancer activity, we tested them *in vitro* for their potent anti-EMT activity in RPE cells and found that EGCG and curcumin inhibited EMT in RPE cells.

Proliferation assay showed that EGCG, curcumin and lycopene inhibited the proliferation of ARPE-19 cells induced by TGF-β1. Migration of RPE cells induced by TGF-β1 was reduced by EGCG and curcumin, but not lycopene. TGF-β1-induced migration in ARPE-19 cells was dependent on MMP-2. Doxycycline, an inhibitor of MMP-2, reduced the migration of ARPE-19 cells[11]. Lycopene did not inhibit MMP-2 and, therefore, possibly did not inhibit migration of RPE cells. Previous reports documented that EGCG and lycopene inhibited migration and proliferation of ARPE-19 cells induced by platelet-derived growth factor-β which was independent of MMP-2[25,26].

VEGF-A is a proangiogenic factor and is also implicated in maintenance of endothelium and other cells like neuronal and immune cells[27]. However, it also plays an important role in tumour metastasis by inducing EMT[28]. TGF-β1 is an inducer of VEGF-A in RPE cells[29] and the secretion of VEGF-A acts as a positive feedback loop, further inducing EMT. EGCG and curcumin inhibited VEGF-A secretion and are, therefore, capable of inhibiting the feedback loop.

Previous reports have shown that EGCG inhibited EMT in lung cancer[6] cells and nasopharyngeal cancer cells[7], but the mechanism is not elucidated. Curcumin inhibits doxorubicin-induced EMT in breast cancer cells and non-cancerous renal tubular cells[8]. However, the mechanism of EMT inhibition was not through Smad-3 phosphorylation. A report suggested that resveratrol inhibited PVR by inhibiting EMT by blocking acetylation of Smad-4 *in vivo* and *in vitro*[30]. Our previous report suggested the role of Smad-3 phosphorylation in EMT of ARPE-19 cells, wherein Smad-3-specific inhibitor reversed the expression of EMT markers altered by TGF-β1[11]. We have shown earlier that EGCG and curcumin inhibit EMT induced by TGF-β1 in RPE cells through inhibiting Smad-3 phosphorylation, while there is a report of EGCG inhibiting acetylation of Smad-3 in renal tubular cells[6], indicating varying mechanisms of EMT based on the cell type. Lycopene did not inhibit Smad-3 phosphorylation but reduced the proliferation induced by TGF-β1. Hence, this effect of TGF-β1 is probably met through other non-canonical pathways, which were inhibited by lycopene. The study limitation was that the various other possible mechanism of action of the compounds were not explored.

The study thus reveals that EGCG and curcumin inhibited EMT and exhibited anti-MMP-2, antiproliferation and anti-migration activities in RPE cells under TGF-β1-induced conditions. In our previous study, we reported on the anti-EMT activity of chebulagic and chebulinic acidat a concentration of 100 μM[11]. However, EGCG and curcumin showed maximal anti-EMT activity even at a lower concentration of 10 μM.

In conclusion, EGCG and curcumin reversed EMT through inhibition of Smad-3 phosphorylation induced by TGF-β1 in ARPE-19 cells. Lycopene did not prevent EMT in ARPE-19 cells. EGCG and curcumin could be potential molecules for adjuvant therapy in PVR management. Further pre-clinical studies in an animal

model of PVR is required for evaluating the effect of the compounds *in vivo*.

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***Conflicts of Interest*:** None.

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