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In vitro validation of novel drug candidate EGFR inhibitor in treatment of colorectal cancer employing 2D and 3D cell culture techniques

Amrutha Krishnan K[1,3]*, Sudha George Valavi[1,3] & Amitha Joy[2,3]

[1]Department of Applied Science and Humanities, [2]Department of Biotechnology, Sahrdaya College of Engineering and Technology, Kodakara, Kerala, India

[3]APJ Abdul Kalam Technological University, Kerala, India

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Colorectal cancer (CRC) is deemed to be the third leading cause of cancer-related deaths worldwide with an overall mortality rate of 10%. One of the major causes of CRC is overexpression of EGFR, and currently, there are very few effective medications available to target it. This study intends to validate the potency of 2-((1,6-dimethyl-4-oxo-1,4-dihydropyridin-3-yl)oxy)-N-(1H-indol-4-yl)acetamide (E1) which has previously been reported as an effective EGFR inhibitor via *in silico* approaches. Anticancer investigation, toxicity analysis, indirect ELISA studies, clonogenic assay, live-dead assay, Reactive Oxygen Species (ROS) assay, and flow cytometry analysis were conducted. Compound E1 inhibited colorectal cancer cells by 38% at an LC_{50} of 156.0365 µg/mL, while inhibiting normal cells by only 15% at an LC_{50} of 418.3150 µg/mL. Activity of BRAF (downstream antibody of EGFR in the MAPK pathway) was reduced three-fold compared to control samples, demonstrating the compound's efficacy. Colony formation ability of the cancer cells was reduced by four-fold while the ROS contents were declined by 23 times in the presence of E1. Cell cycle arrest was confirmed at G0/G1 phase through flow cytometry analysis. RT-PCR studies also confirmed the EGFR inhibition ability of E1. 3D cell culture validation of E1 established strong anticancer inhibition ability for the compound E1. The nominated compound E1 thus can be considered as a novel and potential EGFR inhibitor.

Keywords: Anticancer evaluation, Toxicity analysis, Indirect ELISA, Apoptosis, Cell cycle arrest, RT-PCR

Colorectal cancer (CRC) is regarded as the world′s third deadliest cancer, which predominantly begins as a polyp (non-cancerous growth), in the mucosal epithelial cells of the colon or rectum of the digestive tract of the human body and progresses to invasive cancer[1]. Over years, the rate of occurrence of CRC is alarmingly rising around the world. According to data from the World Cancer Research Fund International, in 2020, around 1.93 million people were diagnosed with this malignancy, accounting for 10.7% of the total number of new cases[2]. Out of the total CRC cases reported to date, around 70% were caused by the overexpression of the Epidermal Growth Factor Receptor (EGFR), a receptor tyrosine kinase that plays a major role in the production, proliferation, and maintenance of cells in the human body[3]. Various diet and lifestyle factors such as smoking, consumption of alcohol, saturated and N-6 unsaturated fat etc. all lead to the over-simulation of EGFR leading to CRC[3].

Over the years, efforts have been made to develop effective EGFR-targeted cancer therapies. Research groups like Rothenberg *et al.* and Yanagisawa *et al.* have employed EGFR inhibitors like Gefitinib and Osimertinib respectively in their studies to prove the efficiency of them in the treatment of CRC[4,5]. However, the effectiveness of the present EGFR inhibitors is limited by the fact that many malignancies are either resistant to them at first or develop resistance while being treated[6]. Thus, seldom effective tyrosine kinase inhibitors are currently employed as drugs for the treatment of CRC[5-10]. In a world where targeted therapy is gaining popularity, the need of an effective EGFR inhibitor for the treatment of EGFR-induced-CRC is imperative and non-commutable.

The authors have already identified a potential EGFR inhibitor for the treatment of CRC by employing various *in silico* approaches[11]. The study used various computer-aided techniques and revealed that the compound 2-((1, 6-dimethyl-4-oxo-1, 4-dihydropyridin-3-yl)oxy)-N-(1H-indol-4-yl)acetamide (E1) is a prospective drug candidate for the inhibition of EGFR[11].

*Correspondence:
E-mail: krishnamrutha18@gmail.com

In this study, the validation of the compound E1 was performed with the aid of numerous *in vitro* analyses. Anticancer activity and toxicity studies were conducted in HT-29 cell line to determine the efficacy of the compound E1[12-14]. The compound's ability to inhibit EGFR was demonstrated using an indirect Enzyme-linked immunosorbent assay (ELISA) test. Cell apoptosis was analysed using live-dead assay, Reactive Oxygen Species (ROS) assay, and clonogenic assay. Further cell arrests were studied using flow cytometry. Anticancer activity was evaluated using 3D cell culture techniques in this work, which allow the retention of cellular shape and heterogeneity that characterise normal tissues and have a significant impact on gene expression and, consequently, cell behaviour[15-16].

Materials and Methods

Synthesis of 2-((1, 6-dimethyl-4-oxo-1, 4-dihydropyridin-3-yl)oxy)-N-(1H-indol-4-yl) acetamide (E1)

The compound E1 was synthesised by MSPS Pharma Pvt Ltd Hyderabad. The steps involved in the synthesis are as follows

Step 1-Synthesis of 2-chloro-N-(1H-indol-4-yl) acetamide (3)

300 mg of compound 1 and 277 mg of compound 2 were added to 6 mL of acetonitrile (ACN) and mixed well to form a homogeneous mixture. To this, 0.9 mL triethylamine (TEA) was added, and the reaction mixture was stirred at 25°C for 3 hours. The reaction mixture was then concentrated, yielding crude material. Crude was purified by column chromatography on silica 60-120 using ethyl acetate in pet ether (20-30%) as the eluent to obtain 2-chloro-N-(1H-indol-4-yl) acetamide (3).

Step 2-Synthesis of the compound E1:

120 mg of compound 3 was dissolved in 2 mL dimethylformamide (DMF). To this, 238 mg potassium carbonate (K_2CO_3) and 80 mg of compound 4 were added and the reaction mixture was agitated for 6 hours at 80°C. The reaction mixture was subsequently concentrated under decreased pressure to get crude. Crude was purified by reverse-phase column chromatography using an ACN-water eluent (40-50%) to afford E1. Fig. 1 shows the synthesis scheme of the compound E1.

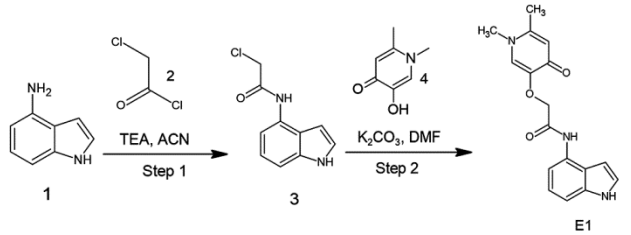

Fig. 1 — Diagrammatic representation of the synthesis scheme of E1

Preparation of cell culture and anticancer evaluation by MTT assay

The seeding culture for the current investigation was made up of HT-29 (Human Colon Cancer) cells obtained from the National Centre for Cell Sciences (NCCS) in Pune, India and maintained in Dulbecco's modified Eagles medium. To assure sterility, 1mg of E1 was weighed and diluted in 1mL of DMEM before being filtered through a 0.22 µm Millipore syringe filter. The solution was serially diluted five times using a two-fold dilution method: 100µg, 50µg, 25µg, 12.5µg, and 6.25µg in 500µL of DMEM. After removing the growth medium, 100µL of each solution was put in triplicates to the respective wells of 24-hour grown cell lines. The cells were then incubated at 37°C in a humidified 5% CO_2 incubator. Non-treated control cells were also preserved.

After 24 hours of incubation, cell viability was assayed using the MTT assay according to the available protocol[17]. The percentage viability of the cells was further calculated using the formula:

$$\% \text{ viability} = \frac{\text{Mean OD sample}}{\text{Mean OD control}} \times 100$$

Where OD is the optical density at 540nm of the solution measured using a spectrophotometer. The images indicating changes in cell viability were then captured using an inverted phase-contrast microscope.

Cytotoxicity determination by MTT assay

The cytotoxic effect of E1 was determined on L929 Mouse fibroblast cell lines. The cytotoxicity evaluations were conducted using the MTT assay according to the same protocol described above. The percentage viability obtained from subsequent calculations was used to determine the minimum lethal concentration of E1 in normal fibroblast cell lines.

Estimation of EGFR inhibition potency by indirect ELISA test

The compound of interest E1, is predicted to be an EGFR inhibitor. Hence to confirm its inhibitory activity, the expression of a downstream gene (BRAF) in the pathway was estimated. For that, the cells were treated with E1 for 12 hours which was assigned as test, and non-treated cells were taken as control. Wells were cleaned and blocked with phosphate-buffered saline (PBS) before the primary antibody (BRAF) was added. After 2 hours of incubation at 25°C, the primary

antibody was removed, and the wells were again washed. Subsequently, horseradish peroxidase (HRP) conjugated secondary antibody was added and incubated for 1 hour at 25°C[18]. The wells were again washed with PBS, added chromogen, and kept for 30 minutes at 25°C (dark incubation). Finally, the reaction was halted by adding 5N hydrochloric acid and absorbance was noted at 415nm.

The activity of antibody was calculated using the formula

$$\text{Activity of antibody} = \frac{\text{OD value}}{\text{Protein concentration}}$$

Estimation of colony formation ability by clonogenic assay

The clonogenic experiment was carried out using the E1-treated cells from the stock of 1mg/mL in a 6-well plate. Agarose solutions at 1% and 0.8% were prepared and stored in a 560°C water bath. An equal volume of 1% agarose and DMEM was used to form the bottom layer, which was left to solidify. A mixture of 0.8% agarose and DMEM was used to make the top layer. The transfected cells were trypsinised and gently applied to the top layer. The plates were incubated for 14 days for uninterrupted colony development. The colonies were fixed with 4% formaldehyde for 2 hours before being stained with 0.005% Crystal violet for 30 minutes. Only colonies with at least 50 cells were counted in each assay. The procedure was repeated three times for precise results. The untreated cells served as the negative control.

Estimation of apoptosis by live-dead assay

The incubated HT-29 cells (both treated and control) were rinsed with cold Phosphate buffered saline (PBS) and dyed for 10 minutes at 27°C with a combination of DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EtBr). The dyed cells were rinsed twice with 1× PBS and consequently examined with a fluorescence microscope in blue filter.

Estimation of reactive oxygen species concentrations by ROS assay

The incubated E1-treated HT-29 cells were washed with PBS before being treated with 50µL of 2'-7'-Dichlorodihydrofluorescein diacetate (DCFDA) followed by an incubation of 30 minutes. Afterwards, the surplus dye was removed using PBS, and the fluorescence was photographed under a fluorescent microscope. The fluorescence was measured at 470 nm (excitation) and 635 nm (emission) and reported in arbitrary units. The untreated cells were used as the control.

Estimation of cell cycle arrest by flow cytometry analysis

The cell cycle analysis was performed using the MUSE cell cycle kit. The kit utilises a ready-to-use reagent containing the nuclear DNA intercalating stain propidium iodide (PI), which distinguishes cells at different stages of the cell cycle by measuring differential DNA content in the presence of RNase to enhance the specificity of DNA staining in each phase (G0/G1, S, and G2/M).

HT-29 treated with LC_{50} concentration of E1 was added and then incubated at 37ºC in a humidified 5% CO_2 incubator for 24 hours. Non-treated control cells were also maintained in similar conditions. The treated and control cell culture samples were mixed with PBS in a polystyrene tube and centrifuged at 3000 rpm for 5 minutes. With the cell pellet intact, the supernatant was then removed. The pellet was then resuscitated in the remaining PBS. Drop by drop, the reconstituted cells were introduced to the tube holding 1mL of ice-cold 70% ethanol while being vortexed at a medium pace and subsequently frozen the tube at -20°C. Following the overnight incubation, the samples were centrifuged at 3000 rpm for 5 minutes at 25°C. The supernatant was removed, and 250µL PBS was added to the pellet. Then the centrifugation was carried out once more with the same conditions as before. The supernatant was removed, 250µL of cell cycle reagent propidium iodide (PI) was added to the pellet and incubated at dark for 30 minutes. This was then analysed using a flow cytometer and was classified by comparing with reference to untreated control cells.

Quantification of gene expression by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Extraction of gene expression data involves various steps such as cell harvesting, RNA isolation, DNase treatment to remove co-purified contaminating DNA, cDNA synthesis, and qPCR. Total RNA was extracted using the total RNA isolation kit (Invitrogen) according to the guidelines provided by the maker. 1 ml of TRIzol solution was added to the cell culture well plate and incubated for 5 minutes, disrupting cells and releasing RNA. The contents were then transferred to a brand-new, sterile eppendorf tube. After adding 200µL of chloroform at the ambient temperature, the mixture was centrifuged at 14000 rpm for 15 minutes at 4°C. The aqueous layer was extracted, then 500µL of 100% isopropanol was added. It was incubated for 10 minutes at room temperature before being centrifuged at 14000 rpm

for 15 minutes at 4°C. When combined with isopropanol, RNA precipitates as a white pellet on the side and bottom of the tube. The supernatant was disposed, and the pellet was rinsed with 200µL of 75% ethanol. It was then centrifuged at 14000 rpm for 5 minutes at 4°C in a cooling centrifuge (Remi CM12). The RNA pellet was dried then suspended in Tris-EDTA (TE) buffer.

cDNA preparation kit (G Biosciences) was utilised to synthesise complementary DNA (cDNA) templates. An RNAse-free tube was filled with approximately 5µL of RT Easy mix, 0.5µL of oligodT, and 2µL of RNA template (0.5µg of total RNA). After that, sterile distilled water was added to bring the reaction volume to 10 µL. Gently pipetting the fluid up and down allowed it to be combined. The eppendorf master cycler, a thermal cycler, was configured to synthesise cDNA. The cycling was done for 20 minutes at 42ºC (cDNA synthesis) and 5 minutes at 85ºC (inactivation).

Subsequently, Real-Time qRT-PCR analysis was carried out using SYBR Green Master Mix (G Biosciences) using Lightcycler 96 (Roche). All reactions were performed in triplicates and data were analysed according to double differentiation method (using Light Cycler 96 SW 1.1 Software). The details of primer sequences used is summarised in Table 1.

Estimation of anticancer activity by 3D cell culture technique

The protocol by Carroll *et.al* was referred for the spheroid generation[19]. The confluent monolayer of cells, which is 2 days old, were trypsinised and suspended in 10% growth medium followed by an addition of 40µL of a 10 mg/mL DNAse. The cells were centrifuged, and the supernatant was discarded. The cells were resuspended in 2mL of complete tissue culture medium and hanging drops were formed by carefully pipetting 20µL cell suspension into the lid of the petriplate. The bottom of the petriplate was filled with 5 mL of PBS which acts as a hydration chamber. The lid was inverted onto the PBS-filled bottom chamber and incubated at 37°C/5% CO_2/95% humidity. The drops were monitored daily and incubated until aggregates have formed. Once aggregates were formed, they were transferred to Thermo Scientific™ Nunclon™ Sphera™ low attachment plates containing 3 mL of complete medium and incubated at 37°C and 5% CO_2 until spheroids were formed.

1mg of E1 was dispersed in 1mL DMEM using a cyclomixer and then filtered through 0.22µm Millipore syringe filter to remove any kind of impurities. Once the spheroids were formed, the anticancer evaluation was conducted by MTT assay as described in the previous section.

The entire plate before and after the treatment with E1 was visualised under an inverted phase contrast tissue culture microscope and the images were recorded.

Results

Anticancer evaluation studies

The dose-response curves depicting the anticancer effect of E1 on HT-29 cell line is given in Fig. 2. The photographic images of the cells with the treatment of the E1 are shown in Fig. 3. The LC_{50} value of the E1 sample in HT-29 cell line was calculated using ED50 PLUS V1.0 software and was observed to be 156.0365 µg/mL.

Cytotoxicity evaluation studies

The dose-response curves depicting the cytotoxicity effect of E1 on L929 cell line is given in Fig. 4. The photographic images of the cells with the treatment of the E1 are shown in Fig. 5.The LC_{50} value of the E1 sample in L929 cell line was calculated using ED50 PLUS V1.0 software and was observed to be 418.3150 µg/mL.

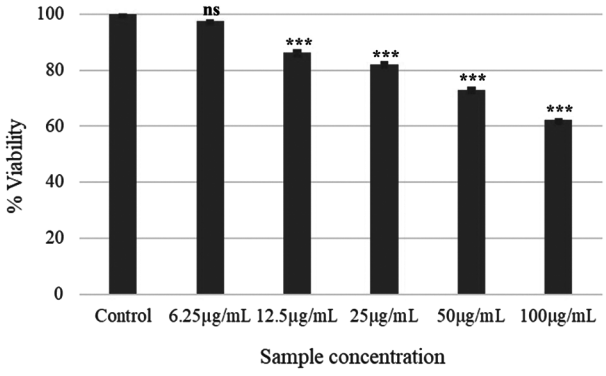

Fig. 2 — Graphical representation depicting the anticancer effect of E1 on HT-29 cell line (P< 0.001 compared to control).

Table 1 — Primer sequences used in RT-PCR

Oligo Name	Forward		Reverse	
	Sequence (5′–3′)	Tm	Sequence (5′–3′)	Tm
H-GAPDH	ACTCAGAAGACTGTGGATGG	57.3	GTCATCATACTTGGCAGGTT	55.3
H BRAF	GGCAGAGTGCCTCAAAAAGAA	57	AACCAGCCCGATTCAAGGA	58

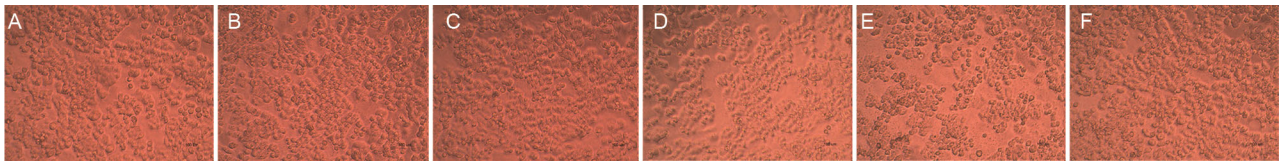

Fig. 3 — Photographic images of HT-29 cells treated with E1 sample of dosage (A) 6.25% (B) 12.5% (C) 25% (D) 50% (E) 100% and (F) 0% (control sample).

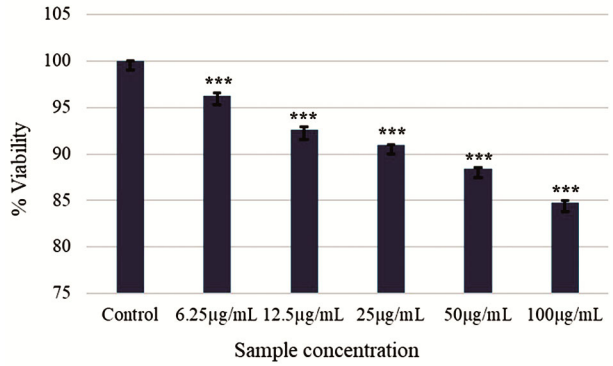

Fig. 4 — Graphical representation depicting the cytotoxicity of E1 on L929 cell line ($P< 0.001$ compared to control).

Downstream protein activity evaluation studies

In the indirect ELISA analysis, the downstream antibody of EGFR pathway, BRAF, is first attached to antigen restrained on a solid phase, and subsequently the antigen is detected indirectly by the HRP conjugated secondary antibody[20-21]. The BRAF protein activities with and without the treatment of HT-29 cells with E1 is given in Table 2.

Colony inhibition evaluation by clonogenic assay

The number of colonies counted after 14 days of experiment indicates the ability of the cells to colonise, which is a hallmark of cancer. The control cell sample had 568 colonies after the clonogenic experiment, while the treated cell sample had only 134 colonies. This suggests that upon exposing with E1, the cell development was suppressed indicating the efficiency of E1. Fig. 6 shows the photographic images of both colonies.

Apoptotic assay and analysis of cell vitality

The distinction between apoptotic and vital cells was accomplished in live-dead assay using the DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EtBr)[22]. AO is taken up by both live and dead cells and emits green fluorescence when interspersed into double-stranded nucleic acid (DNA). EtBr is only absorbed by unsustainable cells and produces red fluorescence by incorporation into DNA[23]. From the result (Fig. 7), it was observed that in the control sample, no red fluorescence is visible indicating the complete viability of the cells whereas in the E1 sample, the red fluorescence is very high confirming the death of the cells. The compound E1 henceforth has the ability to inhibit colorectal cancer cells in the body.

ROS studies and fluorescence intensity analysis

DCFDA, a fluorogenic dye used to mark the hydroxyl, peroxyl and other ROSs activities within the cell, is used in the ROS analysis. Once diffused into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound 2′,7′-dichlorodihydrofluorescein (H2DCF), which is later oxidized by ROS into 2′,7′-dichlorofluorescin (DCF) which shows green fluorescence when excited with a blue filter[24]. From the ROS assay, it was observed that the fluorescence intensity of the control sample was 326.63 AU while that of the E1 sample 7560.85 AU. Hereby it was inferred that E1 kills and inhibits the HT-29 cells, releasing various ROSs in the process, which causes the high fluorescence in the treated sample. Fig. 8 shows the photographic images of the fluorescence of both the samples.

Cell cycle distribution studies by flow cytometry

DNA content is an indicator of cellular maturity in the cell cycle. Cells in the G0/G1 phase have one unit of DNA; cells in the S phase duplicate DNA, increasing its content proportionately to the S phase; and cells in the G2/M phase have two units of DNA, which is twice that of the G0/G1 phase. Due to the candour of this analysis, it most frequently used for the analysis of DNA content to obtain the cell cycle distributions in tumors or other invasive malignancies. When agents disrupting the cell cycle progression are applied *in vitro* or *in vivo* to asynchronously growing cells, the cells gather in the phase of the cell cycle where the perturbation is greatest. This results in an increase in the relative proportion of cells in that phase of the cycle on DNA content frequency histograms[25,26].

The DNA distribution histogram of HT-29 cells in the absence and presence of E1 sample is shown in

Table 2 — BRAF protein concentrations in E1 and control samples

Sample	Absorbance at 415nm	Protein concentration	Activity units/mg protein
E1	0.117	0.461	0.255
Control	0.302	0.441	0.685

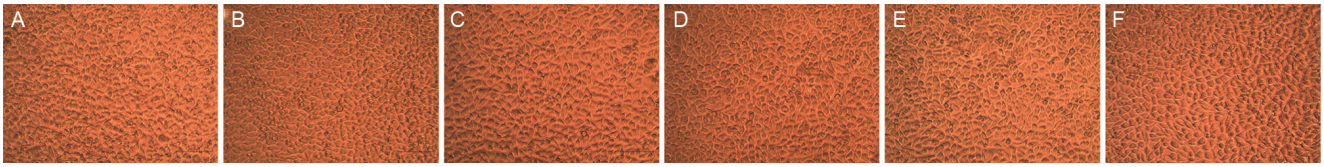

Fig. 5 — Photographic images of L929 cells treated with E1 sample of dosage (A) 6.25% (B) 12.5% (C) 25% (D) 50% (E) 100% and (F) 0% (control sample).

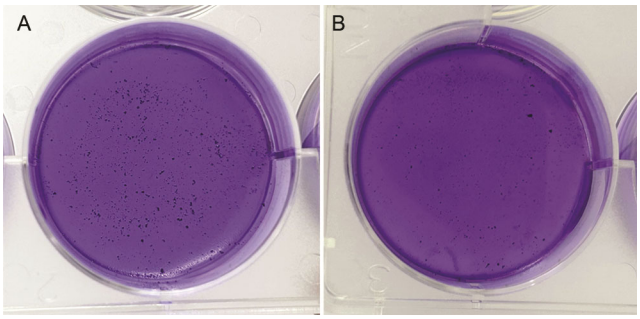

Fig. 6 — Photographic images of colonies in (A) control and (B) E1 sample.

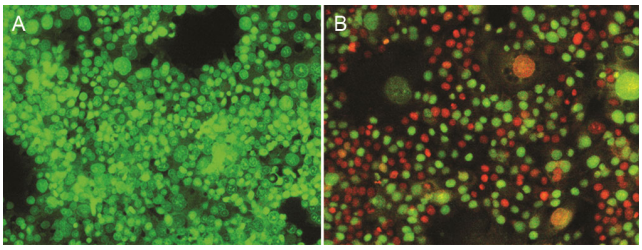

Fig. 7 — Photographing images depicting the viability of the cells analysed (A) control (B) E1 sample.

Fig. 9. In the control sample, the percentage in the cell in the G0/G1 phase was 47.6%. On the other hand, after treatment of cells with E1, the percentage in the cell in the G2/M phase increased to 55.5%. This was accompanied by a corresponding reduction of the cell percentage in the S phase from 18.8% (control) to 4.6% (treated sample) and in the G2/M phase from 28% (control) to 9.1% (treated sample). Therefore, it was confirmed that the cell cycle was arrested at G0/G1 phase.

Gene expression studies by RT-PCR

RT-PCR analysis in this study involved assessing BRAF gene expression, a downstream target downregulated by EGFR inhibition. Upon treatment with E1, the expression of BRAF was found to be -3.5842-fold decreased compared to the untreated control sample, indicating that the EGFR concentration is suppressed. The graph depicting the protein fold change is given in Fig. 10.

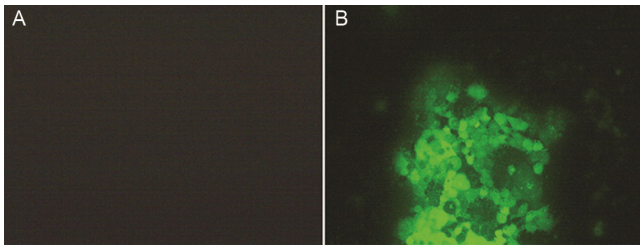

Fig. 8 — Photographic images exhibiting the fluorescence of (A) control and (B) E1 sample.

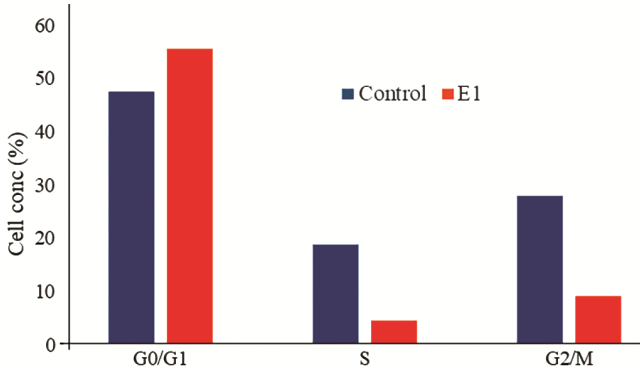

Fig. 9 — Flow cytometry graph depicting the cell concentrations in control and E1 samples in various cell cycle phases ($P< 0.001$ compared to control)..

Anticancer evaluation using 3D cell culture

The 3D cell culture technology is an *in vitro* system that can replicate *in vivo* cell behaviours more accurately and yield more consistent outcomes for *in vivo* experiments[27]. The morphological and physiological characteristics of cells in a 3D culture environment are distinct from those of cells in a 2D

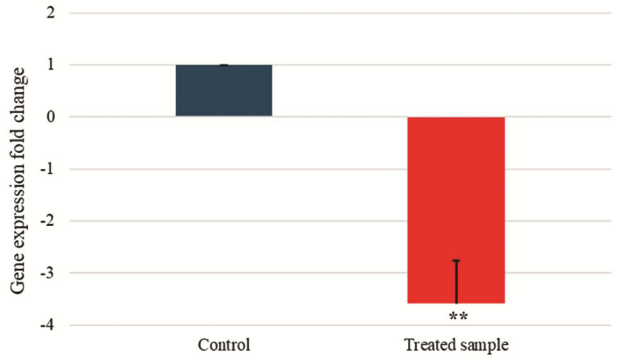

Fig. 10 — Graph depicting the protein fold change in RT-PCR studies (P< 0.01 compared to control)..

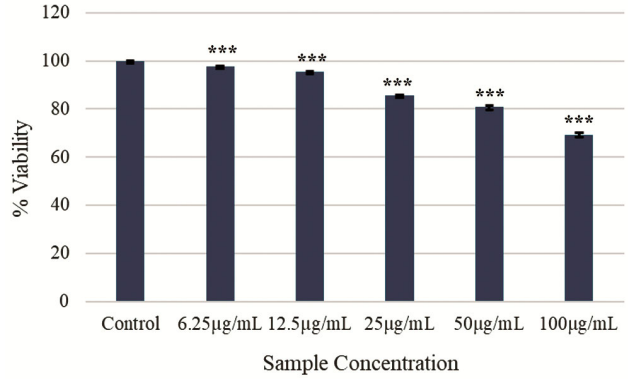

Fig. 11 — Graphical representation depicting the anticancer effect of E1 on 3D cultured HT-29 cell line (P< 0.001 compared to control).

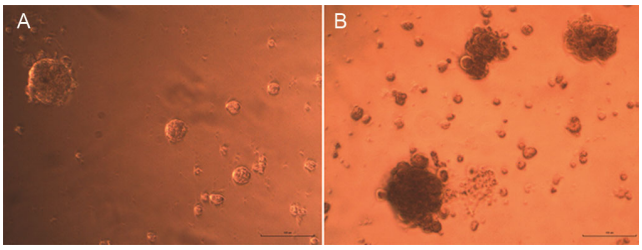

Fig. 12 — Photographic images of spheroids (A) without treatment and (B) with treatment of E1.

culture environment, making it a valuable tool for comprehending the alterations, interactions, and cellular and molecular signalling that occur during malignant transformation[27-29].

The dose-response curves depicting the anticancer effect of E1 on 3D cell cultured HT-29 cell line is given in Fig. 11. The photographic images of the 3D spheroids with and without the treatment of the E1 are shown in Fig. 12. The LC_{50} value of the E1 sample in HT-29 spheroids was calculated using ED50 PLUS V1.0 software and was observed to be 160.809 µg/mL.

Discussion

From the anticancer activity studies, it was observed that the growth of HT-29 cells was inhibited as the amount of E1 administered was linearly increased. The control sample with no E1 administration had shown 100% cell viability whilst the sample administered with 156.0365 µg/mL (100%) had only 62.13% cell viability. As the E1 sample was added, the cytoplasmic shrinkage of HT-29 cells was observed along with the loss of normal cellular architecture. The cell lyses was observed to be maximum when the E1 concentration was 100 µg/mL[30,31]. Hence, it was established that the compound E1 has the ability to inhibit the growth of HT-29 colorectal cancer cells.

Similarly, toxicity studies showed that not much change in the viability of L929 cells were observed in the sample with 100% E1 than from the control sample. The viability of the non-cancerous L929 cells were calculated to be favourable (84.78%) even with the administration of 156.0365 µg/mL of E1. This proved that while compound E1 destroys cancerous cells, it is highly unlikely to be toxic towards the normal cells.

The results of the indirect ELISA test showed that the activity of the BRAF antibody in the non-treated cells (control) was 0.685mg, while that in the E1-treated cells was 0.255mg. The activity of the protein dramatically reduced when the cells were exposed to E1, demonstrating the EGFR inhibition capacity of the compound E1. The clonogenic assay established that the cancer cells treated with E1 had a four-fold drop in colony-formation ability compared to the control sample, while the ROS studies showed that the ROS content in E1 sample was 23 times less than that of the control sample. The apoptosis assay further demonstrated the viability of cells in the E1 and control samples, thereby confirming the efficacy of E1 in inhibiting cancer cell growth. The flow cytometry revealed that the cell cycle is arrested in G0/G1 phase, and the cell division has been controlled. The RT-PCR studies also revealed that the downstream protein concentration is reduced during the administration of E1, indicating the inhibition of EGFR during the process.

The development of 3D cell-based systems *in vitro* can more realistically mimic in vivo cell behaviours and yield more consistent outcomes that are on par with those from in vivo tests. The morphology and physiology of cells grown in 3D culture differ from those in 2D culture. This difference in cell growth affects the spatial arrangement of cell surface

receptors interacting with neighbouring cells and imposes physical constraints on cells, which, in turn, affects gene expression and cellular behaviour, much more similar to cells *in vivo* than *in vitro*[27]. The studies using the 3D cell culture technique revealed that the compound E1 had an activity of 160.809 µg/mL, which is nearly identical to the results obtained with the 2D cell culture approach. The spheroids were healthy and intact before treatment with E1. On the other hand, with the addition of E1, the spheroids became shattered, and the cells decomposed. This proves the efficiency of E1 as an anticancer agent.

This study, however, only employs a variety of *in vitro* methods to assess the effectiveness of E1 on CRC cells. To ultimately ascertain the effectiveness of E1 for EGFR inhibition in the treatment of CRC, additional *in vivo* research must be conducted.

Conclusion

CRC, considered to be the second most deadly type of cancer, is mainly caused by the overexpression of EGFR. Since the existing drugs have various drawbacks of its own, there is a constant need to identify new prospective medications. The authors previously identified a potential EGFR inhibitor, E1, using various *in silico* techniques. This work aimed to in vitro validate the compound E1 as an effective EGFR inhibitor. Anticancer evaluation studies revealed that E1 has an LC50 of 156.0365 µg/mL, at which approximately 38% of HT-29 cells were inhibited. Meanwhile, the toxicity studies showed that E1 poses no danger to healthy cells, with an LC50 of 418.3150 µg/mL, which is 2.7 times higher than the LC50 for cancer cells. Furthermore, the indirect ELISA test also revealed that the activity of downstream protein of EGFR pathway, BRAF, was also reduced 3-fold, which also testified the EGFR inhibition capability of E1. The clonogenic assay, live-dead assay and ROS assay all demonstrated the efficiency of E1. The flow cytometry analysis further revealed cell cycle arrest in cancer cells in the presence of E1. Eventually, the 3D cell culture analysis also demonstrated the efficacy of E1, yielding results equivalent to those from 2D cell culture. Therefore, the compound E1 is proved to have the ability to inhibit EGFR, which in turn makes it a novel potential drug candidate for the treatment of colorectal cancer. Additionally, the efficacy of this compound has to be confirmed by testing in the animal models.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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