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Research Article

Curcumin loaded electrospun poly (2-hydroxyethyl methacrylate) p(HEMA) nanofibers as Antioxidant and Anticancer agents

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Abstract

Curcumin loaded poly(2-hydroxyethyl metharcylate) p(HEMA) nanofiber were prepared using electrospinning technique. The curcumin loaded nanofiber induced dose and time based cell proliferation on A375 melanoma cells. Good free radical scavenging activity were exhibited by DPPH and ABTS methods. Apoptotic activity at 200µg concentration at 192 hrs was effective by DAPI staining method. Expression of cyclin dependent kinase (CDKN2A) gene was quantified by real time quantitative PCR and mRNA expression was up regulated. Hence the results shows that curcumin loaded p(HEMA) nanofiber exihibit good anticancer and antioxidant property.

Keywords: CDKN2A, Curcumin, DPPH, ABTS, p(HEMA) nanofiber

Introduction

Cutaneous malignant melanoma is a complex and heterogenous disease that has some degree of inherited predisposition.[1] CDKN2A a cyclin dependentant kinase inhibitor 2A is a gene responsible for the disease due to some deletion or mutation in 9p21 chromosomal region. CDKN2A codes for cell cycle regulator proteins $p16^{INK}$ and $p14^{ARF1,2}$. Marina cartello et al have shown involvement of CDKN2A in melanoma development and indicated that around 92% of melanoma cell lines had some mutations in CDKN2A gene[3]. J.Lang et al studied CDKN2A mutations 32 Scottish family with cutaneous melanoma resulting in 22% with CDKN2A mutations[4].

Curcumin a yellow pigment phenolic compound isolated from *curcuma longa* has anticancer, antioxidant, antibacterial, anti-inflammatory effects. In our earlier studies we have shown that curcumin loaded p(HEMA) nanofibers can be used as alternate drug for multiresistant organisms[5]. curcumin has anticancer effects like cell cycle arrest, suppression of oncogene and promotion of tumor suppressor genes[6] and also induces apoptosis and suppression of metasis of cancerous cells[7].

p(HEMA) is a biocompatible and biodegradable hydrophilic polymer[8]. p(HEMA) has many useful properties like low toxicity, high water content etc...[9]. In our previous study we have shown electrospun p(HEMA) as suitable scaffold for tissue engineering applications[10]. p(HEMA) has been used as scaffold for myocardial tissue regeneration, controlled and sustained release[11,9,12]. Paclitaxel loaded p(HEMA) bamboo cellulose nanofiber composite proved to be effective matrix for cancer and wound healing[13].

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Electrospinning is versatile technique which requires high voltage,small diameter needle and collector with ground[14]. Polymer solution is electrically charged by providing high voltage. The aim of the study is to prove curcumin loaded p(HEMA) nanofibers induces cell death, exihibit antioxidant activity and up regulate CDKN2A gene expression.

Materials and methods

Poly (2-hydroxy ethyl methacrylate) of average molecular weight (Mw=10,00,000),PCR primers and Curcumin was purchased from Sigma Aldrich, Bangalore, India. Ethanol was purchased from Hayman limited England. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and the supplementary antibiotics for tissue culture were purchased from Himedia, Mumbai, India.

Electrospinning of polymer fibers

Solutions of p(HEMA) with curcumin were prepared as described in our earlier study[5] and nanofibers were prepared using an electrospinning apparatus designed and developed at the Conducting Polymers Lab, Indian Institute of Technology, Chennai, India[15]. Parameters for electrospinning provided in our earlier study[10] were followed to produce curcumin loaded p(HEMA) nanofibers. The nanofibers were deposited onto an aluminium substrate wound around a rotary drum placed perpendicular to the needle.

Cell viability assay

The A375 melanoma cells were purchased from NCCS pune, and were plated separately in 96 well plates at a concentration of 1×10^4 cells/well. After 24 h, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37ºC. After starvation, cells were treated with different concentrations of test compound for 168 hrs. At the end of the treatment period the medium was aspirated and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 h at 37ºC in a CO_2 incubator. The 50% inhibitory concentration value (IC_{50}) of the crude extracts was identified for normal fibroblast cell line.

The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO and this was mixed properly by pipettting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using Graph pad prism5 software.

Determination of 1,1-diphenyl-2-picrylhydrazylradical (DPPH) scavenging assay

DPPH radical scavenging activity of extract was determine according to the method reported[16]. An aliquot of different concentration of sample solution in methanol was mixed with 2.5 ml of 0.5 mM methanolic solution of DPPH. The mixture was shaken vigorously and incubated for 37 min in the dark at room temperature. The absorbance was measured at 517 nm using UV spectrophotometer. DPPH free radical scavenging ability (%) was calculated by using the formula.

Percentage of inhibition = absorbance of control – absorbance of sample / absorbance of control ×100.

2,2'-azinobis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay

The method of decolourisation of free radical ABTS was performed according to the literature [16] with some modification. The ABTS was prepared by mixing an ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture was allowed to stand for 12–16 h at room temperature in the dark until reaching a stable oxidative state. For each analysis, the ABTS solution was diluted with pH 7.4 phosphate buffered saline (PBS) solution to an initial absorbance of 0.700 ± 0.021 at 734 nm. This solution was freshly prepared for each analysis. For the spectrophotometric assay, different concentration of the extract was added to 3.9 mL of ABTS solution and the absorbance was determined at 734 nm. Results were expressed in % of reduction. All determinations were carried out in triplicate.

Apoptosis measurements and 4'-6-diamidino-2-phenylindole (DAPI) staining

To determine the level of apoptosis in drug treated A375 melanoma cells, DAPI staining was performed as described[17]. Briefly, the cells were seeded onto glass slides and treated with Sukun wood extract for 24 h, 48, 96 and 192 hours. Untreated and treated cells were rinsed with phosphate buffered saline (PBS), fixed with ice-cold 10% trichloroacetic acid, and further washed with cold 70, 80, 90% and absolute ethanol. The cells were permeabilized with Triton-X (10 %v/v) and stained with 1 µg/ml 4'-6-diamidino-2-phenylindole (DAPI) for 3 min. To reduce the background, the stained cells were washed with PBS, cover-slipped with 90% glycerol and observed under a fluorescence microscope (Labomed- Carl zeiss Lens with blue filter Olympus India).

Reverse transcription and Quantitative real-time polymerase chain reaction (qPCR)

Quantitative PCR was performed to quantify the expression of CDKN2A in A375 melanoma cells. Cells were treated with curcumin (10 and 50µl) for 48hrs. Each sample was incubated for 45 min at 45° C, followed by 10 min at 72° C in a Agilent amplicon system (AGILENT Biosystems), the

prepared cDNA was stored in -20° C for further use. The cDNA (1µl) was then amplified in 20 µl of reaction buffer for 40 cycles of denaturation (96°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 30s) using primer sequence as follows: *CDKN2A* (forward-5'-TTCGGCTGACTGGCTGGCCA-3', reverse- 5'AGCTCCTCAGCCAGG TCCAC-3') [18]. GAPDH (forward- 'ATGGGGAAGGTGAAGGTCGGAGTC-3', reverse 5'-CCATGCCA GTGAGCTTCCCGTTC-3') [19]. To normalize the expression of apoptotic gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal reference gene. All measurements were performed in triplicate. Real-time RT-PCR data were represented as Ct values, where Ct was defined as the threshold cycle of PCR when amplified product was first detected. To minimize intra- and inter-assay variability caused by differences in PCR efficiency, the quantity of 5-tissue. The Ct or threshold value of the target sequence is directly proportional to the absolute concentration when compared with the threshold value for reference genes. The relative expression level of target gene were plotted as fold change compared to control and determined by the $2^{-\Delta\Delta ct}$ method [20] a relative quantification algorithm. The factor X by which the amount of the changed gene can be calculated with the formula: $X=2^{-\Delta\Delta ct}$. where ΔΔct =(Ct of target) control- (Ct, of target x GAPDH) sample.

Results

Fabrication Of Curcumin Loaded P(HEMA) Nanofibers For Anticancer Activity

Curcumin loaded p(HEMA) nanofibers were fabricated by the electrospinning process. The identification of functional groups, thermal stability, crystallinity and the surface morphology of the prepared nanofibers are discussed in our earlier studies [5]. The '*in-vitro*' cell viability of curcumin loaded p(HEMA) nanofibers on A375 melanoma cells were tested for 1,10, 50,100 and 200 µg concentrations of curcumin. The decrease in the viability of A375 melanoma cells after treatment with curcumin from the p(HEMA) nanofibers at different concentrations for a period of 24, 96 and 168 h (i.e. Day 1, Day 4 and Day 7) respectively were analyzed by MTT assay. Figure 1. shows the 24 h (day 1) viability of A375 melanoma cells. For 1 µg concentration of curcumin the cell viability was around 86 %, at 10 µg curcumin concentration the cell viability were 81 %, at 50 µg curcumin concentration 69 % cells were viable, at 100 µg curcumin concentration 56 % cells were observed to be viable and 53 % cells were viable at 200 µg curcumin concentration. The IC50 value of the A375 melanoma cells for 24 h period was found to be 86.484 µg/ml.

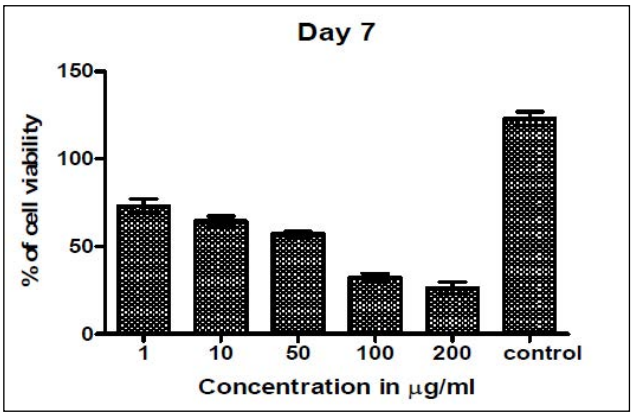

Figure 2.Cell viability of A375 melanoma cells at day 4 for various concentrations of curcumin

Figure 2. shows 96 h (day 4) viability of A375 melanoma cells, for 1 µg curcumin concentration 82 % were viable, at 10 µg curcumin concentration 80 % cells were found to be viable, 62 % cells were observed to be viable for 50 µg of curcumin concentration, 40 % cell viability were seen for 100 µg curcumin concentration and around 37 % cell viability were observed for a period of 96 h (day 4) for 200 µg curcumin concentration. The IC50 value of the A375 melanoma cells for 96 h period was found to be 57. 608 µg/ml.

Figure 3. shows the cell viability of A375 melanoma cells at 168 h (day 7). At 1 µg curcumin concentration 73 % of cells were viable, for 10 µg curcumin concentration 64 % cell viability were observed, 57 % of cells were found to be viable at 50 µg curcumin concentration, at 100 µg curcumin concentration 32 % cells were found to be viable. The cell viability was found to reduce to ~26 % at 168 h for 200 µg concentration of curcumin. The IC50 value of A375 melanoma cells for 168 h was found to be 64.903 µg/ml. Similar pattern of curcumin viability on GBC-SD cells were observed and reported earlier [7]. These results indicated that curcumin loaded p(HEMA) nanofiber had significant effect on A375 melanoma cells which caused cell death. Phase contrast images are shown in Figures 4 - 6. SEM image of curcumin treated A375 melanoma cells are shown in Figure 7. Studies have shown that curcumin induced apoptosis in GBC-SD cells [7]. In the present study, the results showed significant cell death of A375 melanoma cells based on dose and time which indicated that cancer treatment with curcumin loaded p(HEMA) nanofibers inhibited the growth and induced skin cancer cell death.

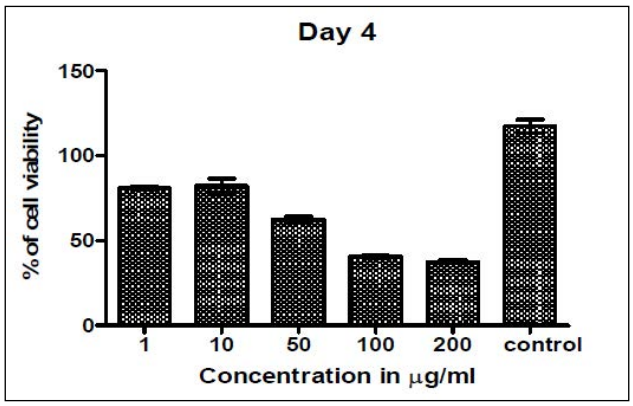

Figure 3.Cell viability of A375 melanoma cells at day 7 for various concentrations of curcumin

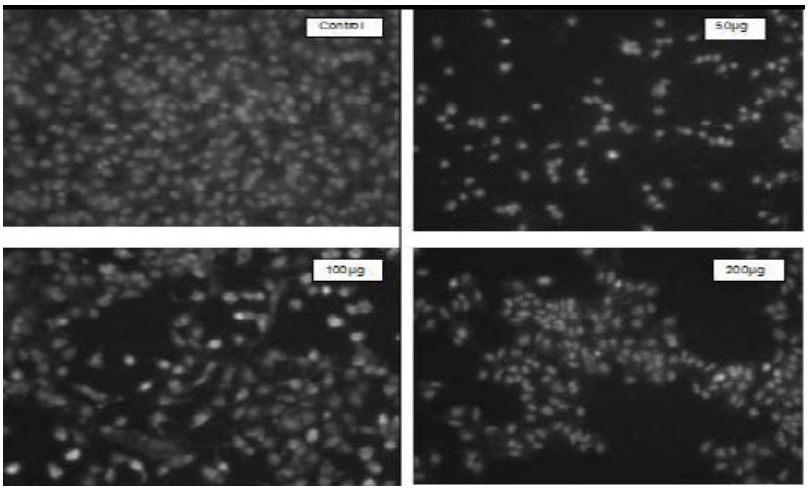

Figure 4.Phase contrast images of A375 melanoma cells at 24 h for various concentrations of curcumin

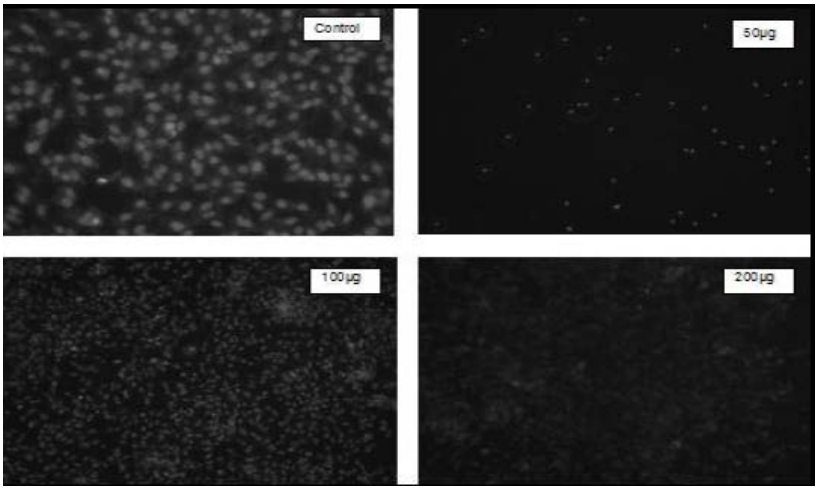

Figure 5.Phase contrast images of A375 melanoma cells at 98 h for various concentrations of curcumin

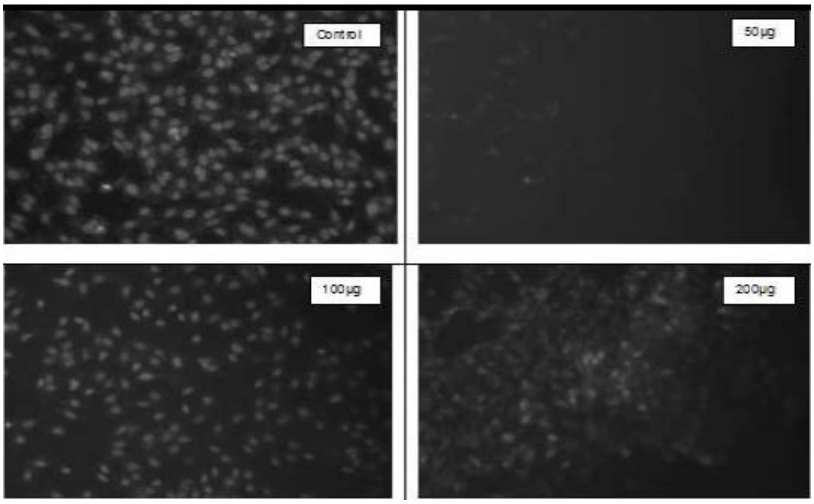

Figure 6.Phase contrast images of A375 melanoma cells at 192 h for various concentrations of curcumin

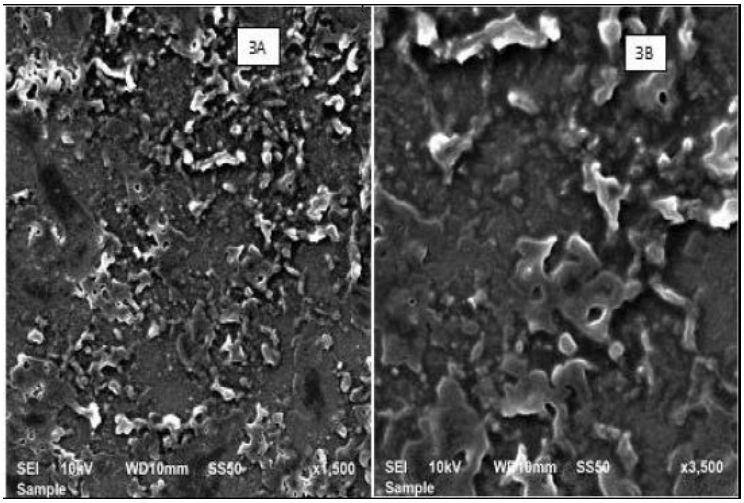

Figure 7.SEM images of curcumin treated A375 melanoma cells (3A) Control (3B) Treated cells

For comparison, neat curcumin cell viability assay using A375 melanoma cells was done for 24, 96 and 168 h respectively at 1, 10, 50, 100, 200 µg concentrations as shown in Figure 8. At 24 h period, the cell viability for 1 µg curcumin concentration were found to be 80 %, for 10 µg curcumin concentration 76 % cells were viable. Cell viability for 50 µg curcumin concentration were 60 %, at 100 µg curcumin concentration, 55 % cells were viable and at about 200 µg curcumin concentration the cell viability were found to be 50 %.

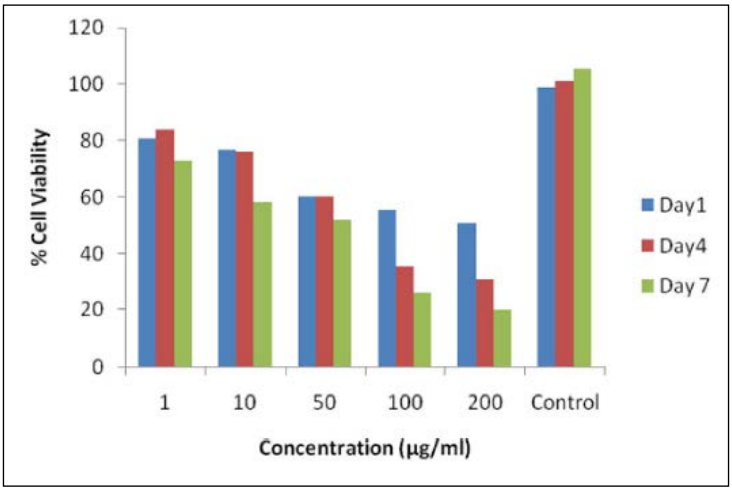

Figure 8.Cell viability of neat curcumin using A375 melanoma cells

At 96 h period, the cell viability for 1 µg curcumin concentration were found to be 83 %. Around 75 % cells were viable for 10 µg curcumin concentration, cell viability for 50 µg concentration was found to be 60 %. At 100 µg concentration 35 % cells were viable and at 200 µg curcumin concentration 30 % cells were viable.

At 168 h period, the cell viability for 1 µg curcumin concentration was found to be 72 %. The cell viability for 10 µg curcumin concentration was found to be 58 % and around 51 % cells were viable at 50 µg curcumin concentration. The cell viability for 100 µg curcumin concentration was found to be 25 % and for 200 µg curcumin concentration, 20 % of cells were found to be viable.

Cell viability for curcumin loaded p(HEMA) nanofiber and neat curcumin was found to be almost the same except a slight decrease in cell death in the case of curcumin loaded p(HEMA) nanofiber. These results show that the curcumin loaded on p(HEMA) nanofiber did not lose its anticancer activity. Hence, even though, the anticancer activity in the case of curcumin loaded nanofiber are slightly less than neat curcumin, the present study confirms the fact that the prepared nanofibrous scaffold can be used in a target site where the oral administration of neat curcumin is not possible[21].

Studies on Apoptotic Activity Using DAPI Staining

Apoptosis of A375 melanoma cells treated with curcumin from p(HEMA) nanofibers was measured using DAPI staining method. Curcumin loaded p(HEMA) nanofiber were treated on A375 melanoma cells with three different concentrations namely 50, 100 and 200 µg at time intervals of 24, 48, 92 and 192 h respectively and stained with DAPI as shown in Figures 9 - 12. When A375 melanoma cells were loaded with 50 µg concentration of curcumin from the p(HEMA) nanofibers, few cells were found to possess structural changes causing cell apoptosis on all the days studied (i.e. 1, 4 and 7 days). However, when the concentration of curcumin was increased to about 100 µg, half of the A375 melanoma cells lost their nuclear DNA and started degrading from the first day. Increasing further the concentration of curcumin to 200 µg concentration from the p(HEMA) nanofibers, the A375 melanoma cell morphology completely changed, its nucleus got completely degraded and the total structure of the cells got destroyed and were found to be dead when compared to the control cells from the first day of study and almost all the cells attained cell death at 192 h period.

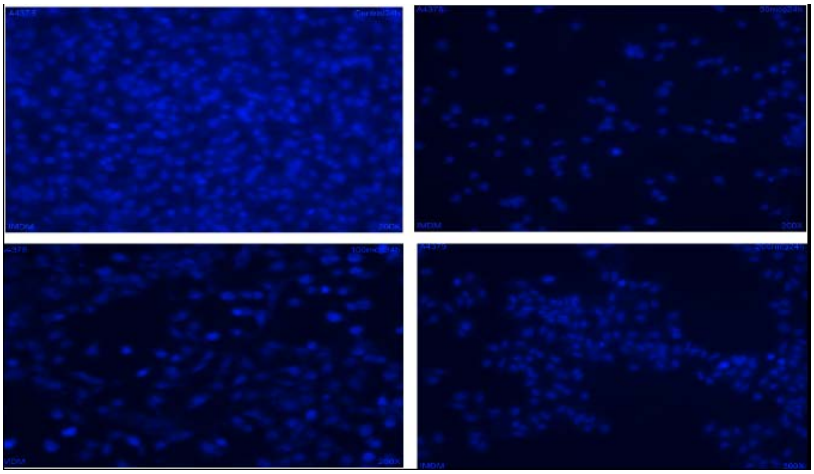

Figure 9.DAPI staining of A375 melanoma cells at 24 h period

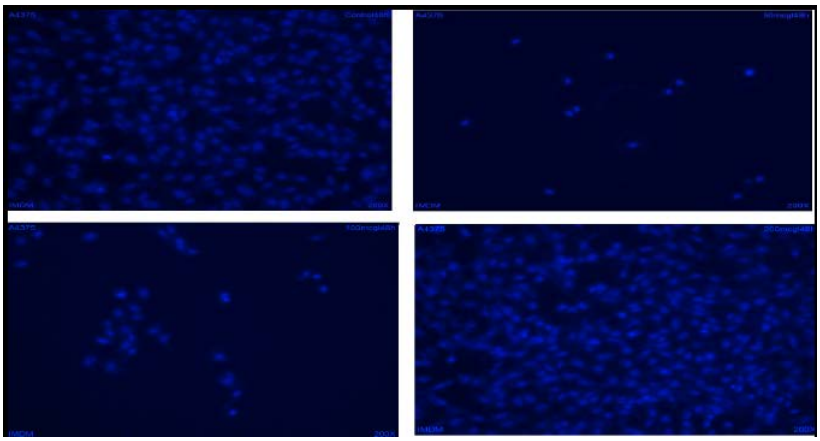

Figure 10.DAPI staining of A375 melanoma cells at 48 h period

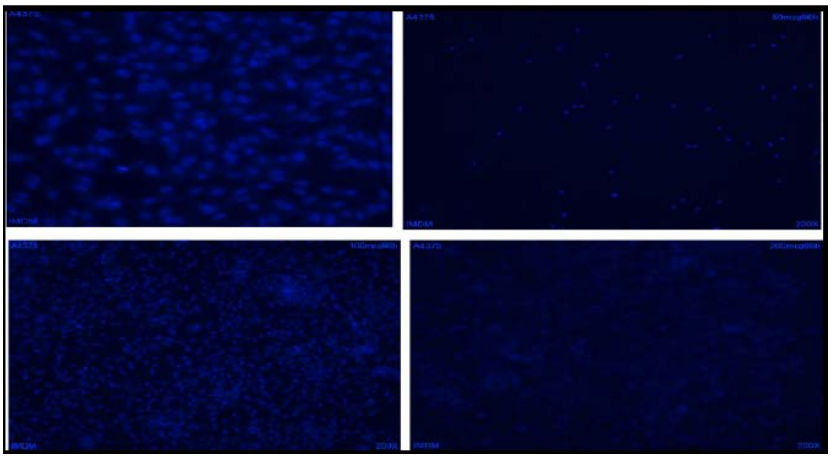

Figure 11.DAPI staining of A375 melanoma cells at 92 h period

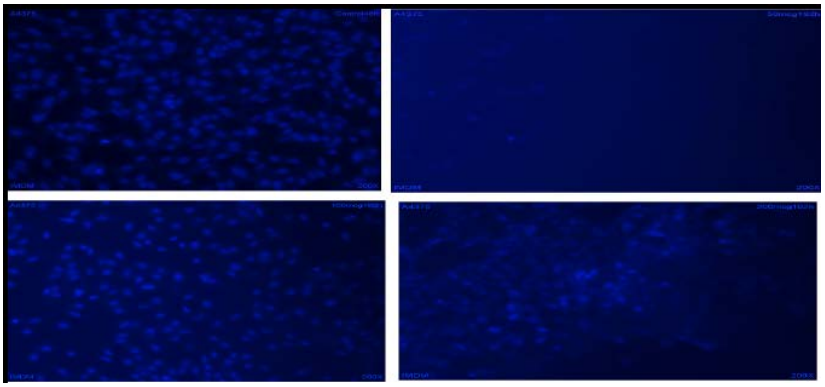

Figure 12.DAPI staining of A375 melanoma cells at 192 h period

Antioxidant Assay Using 2,2-Diphenyl-1-Picryl Hydrazyl (DPPH) Radical Scavenging Analysis

Antioxidant properties are exhibited by the two major mechanisms like hydrogen atom transfer and electron donation[16]. Free radical scavenging activity has been widely evaluated by DPPH assay, as it is one of the very highly sensitive methods to determine the antioxidant activity. At radical site, the methanolic solution of this compound is purple in colour, which absorbs light at a wavelength when reacted with antioxidant it reduces its colour to yellow with no absorbance. Curcumin loaded p(HEMA) nanofibers were found to scavenge DPPH radicals effectively as shown in Figure 13. Curcumin donates a H atom from its methylenic group, it reacts with the electrophilic radicals initially at ionized keto enol moiety. Due to the H- atom transfer phenoxyl radicals are formed. When the concentration of curcumin was increased in the p(HEMA) nanofibers, the radical scavenging activity also increased. Curcumin concentration of 100 µg from the p(HEMA) nanofibers showed 86 % DPPH free radical scavenging activity which showed complete change in colour of the solution to yellow after the reaction. Stable DPPH molecule was formed by the donation of hydrogen atom and the absorbance decreased when DPPH scavenges[22]. The present experimental results showed effective DPPH scavenging activity of curcumin loaded p(HEMA) nanofibers.

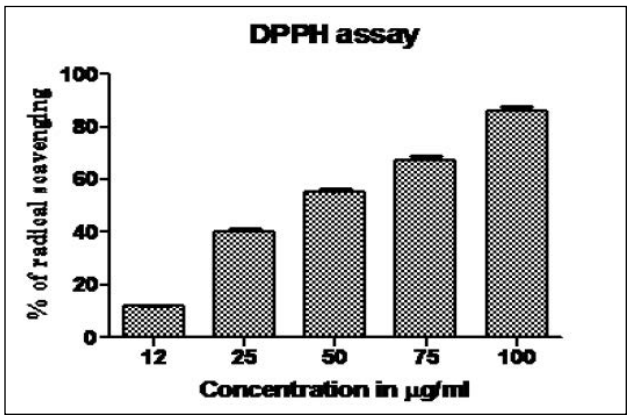

Figure 13.Free radical scavenging activity by DPPH method for curcumin loaded p(HEMA) nanofibers

Antioxidant Assay Using 2,2'– Azinobis – (3-Ethylbenzothiazoline-6-Sulphonicacid) (ABTS) Radical Scavenging Activity

Curcumin loaded p(HEMA) nanofiber showed effective ABTS radical scavenging activity of about 74 % for 100 µg of curcumin concentration as shown in Figure 14. ABTS radical is in green colour, where curcumin suppresses the reaction by electron donation and it inhibits the formation of colour. Absorption of ABTS was found to be decreased for all the concentration of curcumin studied (i.e. 12 to 100 µg/ml) when compared to the previous DPPH method.

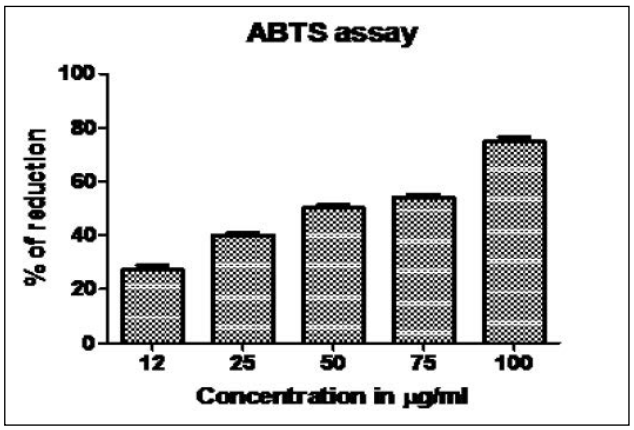

Figure 14.Free radical scavenging activity by ABTS method for curcumin loaded p(HEMA) nanofibers

Effect Of Curcumin Loaded P(HEMA) Nanofiber For Expression Of Cyclin Dependent Kinase Inhibitor 2a (CDKN2a) Gene

Real time quantitative PCR is a reliable method to quantify gene expression and this method was used to quantify the CDKN2A gene expression level. The results of curcumin loaded p(HEMA) nanofiber treatment on A375 melanoma cells showed that the expression of CDKN2A mRNA was upregulated and acted as a tumor suppressor gene and arrested the cell cycle.10 µg/ml of extracted mRNA showed 6.50084 fold change, 50 µg/ml of mRNA showed 36.7743 fold increase of gene expression and 75 µg/ml was found to express 256.111 fold increase of gene expression. Earlier studies have shown that curcumin induced apoptosis of GBC-SD cells[7]. CDKN2A gene mutations were responsible for causing cutaneous melanoma cancer due to cell cycle alteration[24]. In the present study, CDKN2A gene expression was found to be responsible for cell cycle regulation and tumour suppression in melanoma cells as quantified by real time PCR. Curcumin loaded p(HEMA) nanofiber showed up-regulated expression of mRNA of CDKN2A gene which acted as a tumour suppressor and regulated the cell cycle.

Discussion

Curcumin being a natural compound has the ability to inhibit cell proliferation, antioxidant and apoptotic effect on cancer cells. To our best of knowledge our study is first to prove the ability of curcumin loaded p(HEMA) nanofibers to induce cell proliferation, free radical scavenging activity and up regulated expression of CDKN2A on A375 melanoma cells. Many studies have shown that curcumin induces apoptosis in GBC-SD cells[7]. Our study showed significant cell death of A375 melanoma cells based on dose and time which indicates that treatment with curcumin loaded p(HEMA) nanofibers inhibited the growth and induced cell death of skin cancer cells.

Antioxidant property has been exhibited by two major mechanisms like hydrogen atom transfer and electron donation[16]. DPPH and ABTS radical scavenging are very high sensitive method.[22] Stable DPPH molecule was formed by donation of hydrogen atom where absorbance decreased when DPPH scavenges [22]. Our experiment showed effective DPPH scanvenging activity of curcumin loaded p(HEMA) nanofiber as the concentration increased the free radical scavenging activity also increased. Effective ABTS free radical scavenging activity was also observed.

Curcumin at about 200 µg showed cell death apoptotic method. Almost all the cells morphology was alterted and the cells with fragmented nuclei was seen with increase in concentration. Apoptotic activity of cells mostly achieved by cleavage of DNA. Herbal extract showed good apoptotic effect on MCF breast cancer cell line[23].

Earlier studies have shown that curcumin induces apoptosis of GBC-SD cells [7]. CDKN2A gene mutation was responsible for causing cutaneous melanoma cancer due to cell cycle alteration[24]. Real time quantitative PCR is reliable method to quantify gene expression. In our study CDKN2A gene expression responsible for cell cycle regulation and tumour suppressor in melanoma cells was quantified by real time PCR. Curcumin loaded p(HEMA) nanofiber showed upregulated expression of mRNA of CDKN2A gene which acts as tumour suppressor and cell cycle regulator.

Conclusion

To conclude our study suggest that curcumin loaded p(HEMA) nanofiber induces cell death of A375 melanoma cells and exhibits good antioxidant activity by scavenging free radicals,CDKN2A gene mRNA expression was upregulated which indicates that curcumin loaded p(HEMA) nanofiber has an excellent anticancer and antioxidant property.

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