RESEARCH ARTICLE

Purification of Lovastatin from *Aspergillus terreus* (KM017963) and Evaluation of its Anticancer and Antioxidant Properties

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Abstract

Cervical cancer is the second most common malignancy in women worldwide and thus one of the leading causes of mortality in women. Lovastatin, a non polar, anticholesterol drug has previously been reported to exert antitumour activity *in vitro*. In the present study, lovastatin from *Aspergillus terreus* (KM017963) was purified by adsoprtion chromatography and evaluated for its anticancer and anti-oxidant properties with a human cervical cancer cell line (HeLa). Growth inhibitory and proapoptotic effects of purified lovastatin on HeLa cells were investigated by determining its influence on cell numbers, mitochondrial membrane potential (MMP), DNA fragmentation and antioxidant properties in terms of hydroxy radical scavenging effects as well as levels of total reduced glutathione. Cell cycle analysis by flow cytometry (propidium iodide staining) confirmed induction of apoptotic cell death and revealed cell cycle arrest in the G0/G1 phase. Results of the study give leads for the anticancer effects of lovastatin and its potential usefulness in the chemotherapy of cervical cancer.

Keywords: Purification - lovastatin - *Aspergillus terreus* - proapoptotic effects - antioxidant activity

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Introduction

Filamentous fungi such as *Aspergillus*, *Penicillium* and *Talaromyces* are some of the most exploited microbial sources for numerous bioactive compounds such as terpenes, statins, plant hormones, antifungals and anticancer agents. Polyketides represents one of the major classes of fungal bioactive products. One of the significant fungal polyketides with anticancer property belongs to the statin family. Statins are the world's widely prescribed drugs to combat hypercholesterolemia and cardiovascular diseases. In addition, representatives of the statin group are known to possess antifungal properties against Saccharomyces spp., Candida spp., (Macreadie et al., 2006; Thorskov et al., 2013) and also anticancer, anti-oxidant properties. Lovastatin, simvastatin, compactin etc are examples of statins. Commercial production of lovastatin employs *Aspergillus terreus* (Gulyamova et al., 2013).

For a drug to be tested for its bioactive properties, it needs be in the pure form. Most drugs are either purified by solvent extraction or by chromatography, thus meeting the safety standards of effective pharmaceuticals. While solvent extraction procedures are mostly used for easy and fast purification, chromatographic procedures have advantages over solvent extraction concerned to purity. Chromatographic purification can be achieved by adsorption chromatography using silica gel columns or by preparative thin layer chromatography and High Performance Liquid Chromatography (HPLC). Each chromatography process has its own advantages and disadvantages, but adsorption chromatography using silica gel column is widely used because of its easy operation and high purity. Thus this technique of purification is employed in the present study (Ahmed et al., 2009).

Lovastatin, other than its anticholesterol property, has diverse applications in the field of osteoporosis, neuro-degeneration, rheumatoid arthritis, antifungals and also is reported to reduce proliferation of lung cancer cells, breast cancer (MCF-7), liver cancer (HepG2). HeLa cells are human epithelial cervical cancer cell line, and the first human cell line established in culture (Gey et al., 1952) and hence widely used for biological research (Landry et al., 2013). There are few reports available on the antiproliferative effects of lovastatin on cervical cancer (HeLa) cells and there are lesser reports about the effects of lovastatin with respect to its pro apoptotic effects and cell cycle regulation (Thorskov et al., 2013). Therefore the current study was undertaken to understand the proapoptotic effects of lovastatin on human cervical carcinoma cells (HeLa) as model system. Further, the ability of statins to prevent lipoprotein oxidation has also been investigated. Lovastatin, fluvastatin, atorvastatin and simvastatin exert antioxidant effects that protect cells against lipid peroxidation in vitro (Gotto et al., 2001).

In the present study, lovastatin from *Aspergillus terreus* (KM017963) (Praveen et al., 2014) was purified by adsorption chromatography. Purified lovastatin was then evaluated for its proapoptotic and anti-oxidant property using HeLa cells, as an *in vitro* model, using several of

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parameters.

Materials and Methods

Solid state fermentation

Solid-state fermentation was performed in 500ml Erlenmeyer flasks containing 40g of wheat bran as substrate. The substrate was inoculated with spore suspension (107/^{8}ml spores) of *Aspergillus terreus* (KM017963) with relative humidity (RH) of 70% and incubated at 28^{O}C for 8 days (Jaivel and Marimuthu, 2010).

Extraction

After eight days of incubation, the solid substrate was dried at 40^{o}C for 24h, gently crushed and extracted with ethyl acetate by shaking at 180rpm for 2h followed by filtration through whatman No. 1 paper (Reddy et al., 2011). The collected filtrate was concentrated by using rotary vacuum evaporator (Heidolph, Germany)

Chromatographic purification of lovastatin

The crude extract (reconstituted with 5ml of ethyl acetate) of lovastatin was loaded on to pre-packed column (300mm x 18mm) containing silica gel (60-120-mesh size). Elution was started with benzene (100%), followed by Benzene: Acetonitrile (95:5) till Acetonitrile (100%) was reached. Eluent was collected as 1 ml fractions. Each collected fraction was subjected to Thin Layer Chromatography (TLC) to detect the lovastatin fraction (Ahmed et al., 2009).

Thin Layer Chromatography (TLC)

The fractions obtained in column chromatography were applied to a heat activated 20×20 cm silica gel TLC plates. Dichloromethane and ethyl acetate (70:30, v/v) were used as mobile phase. All the plates were observed under Ultra Violet (UV) lamp (254 nm) after developing thrice in the same mobile phase and exposed to iodine vapour. For each TLC run, lovastatin authentic standard (Sigma-Aldrich, Germany) was spotted for Rf value comparison and confirmation (Praveen et al., 2014).

High Performance Liquid Chromatography (HPLC)

For HPLC, a C18 column (250mm x 4.6mm I.D micro metre) with diode array detector was used. Acetonitrile and water (acidified with 1.1% phosphoric acid) (70:30 v/v) were used as mobile phase. The eluent flow rate was maintained at 1.5ml per min and detection carried out at 238nm with injection volume of 20µl (Samiee et al., 2003). The production of lovastatin is expressed in μg/g dry weight substrate (DWS). Purity was confirmed by presence of a single peak of lovastatin. The yield of lovastatin was calculated according to Muthumary and Sashirekha (2007). Mevinolin (M2147) (Sigma-Aldrich, Germany) was used as standard.

Cytotoxicity test by MTT assay

To determine cell viability, MTT based colorimetric assay was performed. Hela cells (1×10^4 cells/well) were cultured in a 96-well plate in DMEM or RPMI-1640 medium at 37°C. Cells treated with culture medium served as a negative control. Cultured cells were treated with lovastatin at different concentrations (0-320μg/ml) and was incubated for 24h. Supernatant from each well was removed and washed twice with Phosphate buffered saline (PBS), 20µl of MTT solution (5 mgml^{-1} in PBS) and 100µl of medium were added, incubated for 4h for the formation of formazan crystals. The resultant crystals were dissolved in dimethyl sulfoxide (100µl) and the absorbance was measured using a microplate reader (Bio-RAD 680, USA) at 590 nm with a reference wavelength of 620 nm. All experiments were performed in triplicate, and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells (Francois and Lang, 1986).

Hydroxyl Radical assay (Deoxyribose assay)

The deoxyribose assay was performed as described by Barry et al. (1987). The reaction volume of 1.0ml contained 5.6mM deoxyribose, 2.8 mM H_2O_2, 40µM $FeCl_3$, 100µM EDTA, and varying concentrations of the purified lovastatin in 2.5mM phosphate buffer, pH 7.4. The reaction was initiated by addition of 0.1mM ascorbic acid and incubated for 90 min at 37°C. After incubation 1ml of TBA (0.7% in 0.05N KOH) and 1ml of TCA (2.5%) was added. The mixture was heated to 100°C for 8 min, cooled and the formed pink colored compound was measured at 532 nm. Corresponding controls were maintained that were devoid of lovastatin. Catechin was used as the reference standard.

GSH assay

Hela cells (10^4cells/well) seeded into 96 well plate and incubated for 24h. Lovastatin was added at varying concentrations and incubated for 24h. Cells were collected by centrifugation at 7000rpm for 5 min at 4ºC and supernatant was discarded. Cell pellet was re-suspended in 0.5ml ice-cold PBS and transferred into a 1.5 ml micro centrifuge tube and centrifuged at 7000rpm for 5min at 4ºC. Supernatant was discarded. Cells were lysed in 80µl ice-cold glutathione buffer and incubated on ice for 10 min. To this reaction mixture, 20µl of 5% Sulfosalicylic Acid (SSA) was added, mixed well and centrifuged at 8000rpm for 10 min at 4^{o}C. Supernatant was used for glutathione assay. To the supernatant, Ellman's Reagent [5,5'-dithiobis-2-nitrobenzoic acid (DTNB)] was added, colour developed was measured at 412nm (Ellman, 1959).

Mitochondrial Membrane Potential Analysis (MMP)

Mitochondrial membrane potential was measured both qualitatively and quantitatively as described by Bishayee et al. (2013). After 48h incubation, control and lovastatin treated (150 µg/ml) cells (10^5cells/well) were fixed with 2% paraformaldehyde and then incubated with 10µM rhodamine-123 for 30min at 37°C in dark. Cells were immediately analyzed using fluorescence microscopy [(OLYMPUS, Japan, omega filters-XF104), (~450nm and >670nm)]. To determine the mitochondrial membrane potential, fluorescence intensity of rhodamine-123 was assessed by a flow cytometer, (BD FACS Calibur, USA). Data obtained were analyzed.

Genotoxicity test by Comet assay

Cultured HeLa cells (10^6cells/well) were maintained in RPMI medium containing 10% foetal calf serum (FCS), 10000 U/l penicillin and 100mg/l streptomycin at 37ºC, 5% CO_2. Cultured cells were treated with different concentrations of lovastatin to check for the induction of DNA damage. Cells were washed briefly with PBS, trypsinized and suspended in medium with FCS to block the trypsin activity. Subsequently, cells were centrifuged for 10 min at 2000rpm. After removal of the medium, the cells were washed with PBS, centrifuged again, re-suspended in PBS and diluted 1:4 with 1% agarose in PBS.

Cell suspension (100μl) was mixed with 0.8% agarose which was added onto fully frosted slides with middle layer and sealed with a coverslip. After lysis 0.5% sodium dodecyl sulphate (SDS) and 33mM ethylene diamine tetra-acetic acid (EDTA) was added and incubated at room temperature (RT) for 40 min. Slides were then incubated for 10min in 1% Tris-Borate-EDTA (TBE) buffer (89mM Tris, 89mM boric acid and 0.2mM EDTA, pH 8.3). Electrophoresis of samples was carried out in TBE buffer at 1 V/cm.

For the alkaline assay, slides were incubated in alkaline lysis buffer (2.5M NaCl, 0.1 M EDTA, 10mM Tris-base, 0.2 M NaOH, 1% SDS, 1% Triton and 10% dimethyl sulphoxide) for 60 min at 4ºC. Subsequently, they were placed in electrophoresis buffer (0.3M NaOH and 1mM EDTA, pH 13) for 25min in order to unwind the DNA and electrophoresed at 1 V/cm for 25min or 2h, after electrophoresis slides were neutralized in Tris buffer (0.4M Tris–HCl, pH 7.5) for 5min (Jafari et al., 2013). Different parameters of DNA damage were analysed by open comet V.1.3 (USA) software.

Cell Cycle analysis

HeLa cells (10^5cells/well) were cultured in a p35 culture plate containing 1 ml of media. After 24h, cells were treated with different concentrations of lovastatin. Control cells were maintained that were not treated with lovastatin. Dimethyl Sulfoxide (DMSO) (1%) in 1mlwell consisting of Dulbecco's Modified Eagle Medium (DMEM) is taken as vehicle control. Above preparations were incubated for 18h. Post incubation, both floating and adherent cells were collected and pelleted at 1500rpm for 5 min at RT and supernatant was discarded. The pellet was resuspended gently in 1X PBS. Cell pellet was fixed overnight at 4°C in 1000 µl of fixing solution (1X PBS in 70% ethanol). Centrifugation was carried at 1500rpm for 5min at RT and supernatant was discarded. Pellet was washed twice with cold PBS (1X). Cells were then incubated for 1h at RT in 500µl of Propidium Iodide (PI) solution containing 0.05mg/ml PI and 0.05mg/ml RNase-A in PBS. The percentage of cells in various stages of cell cycle in lovastatin treated and untreated populations was determined using fluorescence activated cell sorter (FACS) caliber (BD Biosciences, San Jose, CA) and analyzed by flow cytometry (Flow Jo 7.5.5) (Tree Star Ashland OR) (Joany and O'Connor, 2001).

Statistical analysis

All experiments were done in triplicates and statistical analysis was performed by using SPSS, version 20. The statistical difference between mean values were assessed by one way analysis of variance (ANOVA) through Scheffe Post-Hoc test at a significance level of $p<0.01$

Results and Discussion

Lovastatin, by inhibiting HMG-CoA reductase, blocks cholesterol biosynthetic pathway (Seenivasan et al., 2008). Blocking the cholesterol synthesis also results in reduction of isoprenoid moieties of some protein. Thus lovastatin interferes with isoprenylation of proteins, which is a key regulatory step in many biological pathways. Therefore, besides its cholesterol lowering function, lovastatin also have pleiotropic roles in controlling of cell proliferation, apoptosis, survival and differentiation of cancerous cells (Shi et al., 2012).

One of the goals of cancer therapy is to induce apoptosis in cancer cells. Numerous studies suggest that apoptosis is induced by exposure of tumor cells to statins (Jafari et al., 2013). In the present study, we observed that lovastatin produced by *A. terreus* (KM017963) exhibits cytotoxicity on cervical cancer cells (HeLa). This was evaluated by MTT assay, Mitochondrial Membrane Potential assay (MMP) and cell cycle analysis by flow cytometry. Genotoxicity was analyzed by comet assay and antioxidant effects of lovastatin were analyzed by hydroxyl radical scavenging assay and Glutathione (GSH) assay.

Purification by column chromatography

Column chromatographic fractions when screened preliminarily by TLC. The fractions between 11-20

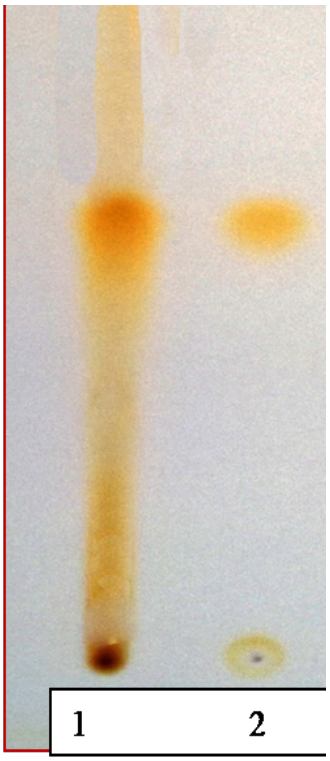

Figure 1. Detection of Lovastatin in 80:20 fraction of Benzene:Acetonitrile by TLC. Lane 1: Purified lovastatin from *A. terreus* (KM017963), Lane 2: Standard lovastatin

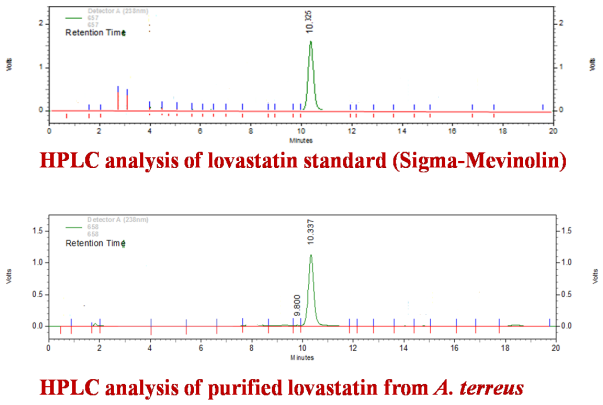

Figure 2. Quantification of Purified Lovastatin by High Performance Liquid Chromatography (HPLC)

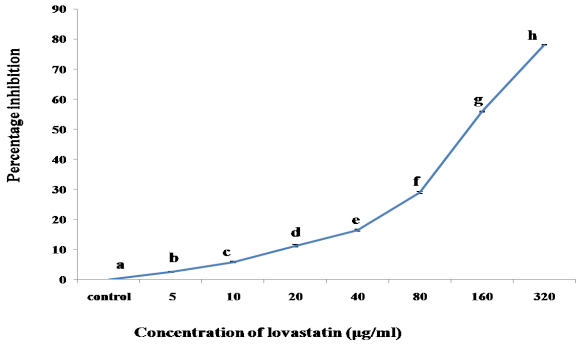

Figure 3. Percentage Inhibition of HeLa Cells Indicating Cytotoxic Effects of Lovastatin

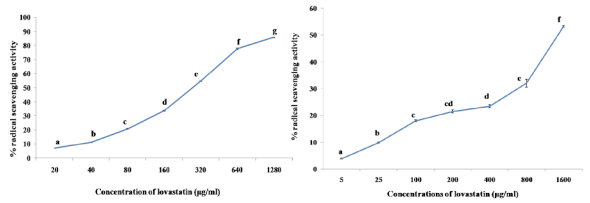

Figure 4. Hydroxyl Radical Scavenging Activity of Standard Catechin and Lovastatin Respectively

obtained through column chromatography using Benzene: Acetonitrile (80:20) ratio, showed the presence of lovastatin (Figure 1). This was further confirmed by HPLC which showed a single, discrete peak (Figure 2). This purified fraction was used for further work i.e., to assess the anti-apoptotic and anti-oxidant property of lovastatin.

Effect of lovastatin on the growth and viability of HeLa cells-MTT assay

The purified lovastatin obtained from the above experiment was taken for all the studies carried out. Lovastatin treatments showed significant dose dependent cytotoxic effect on HeLa cells with IC_{50} value of 160 µg/ml (Figure 3). These results indicated that lovastatin is a strong cytotoxic agent which partially explains its antitumor activity and can be suggested for the use as chemotherapeutic agent (Lucie et al., 2000). The probable reason for cytotoxicity of lovastatin is the attenuation of

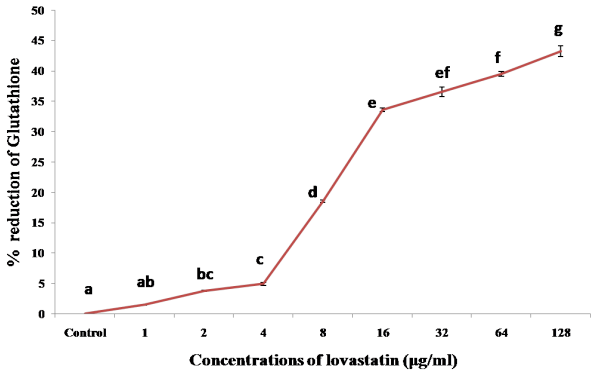

Figure 5. Reduction (Percentage) of Total GSH in Lovastatin-Treated HeLa Cells

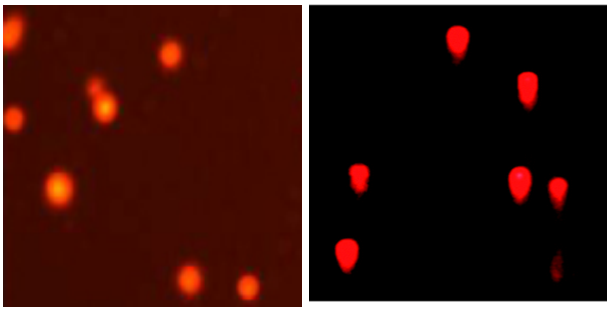

Figure 6. Propidium Iodide Staining of Untreated (Control) and Lovastatin Treated HeLa Cells

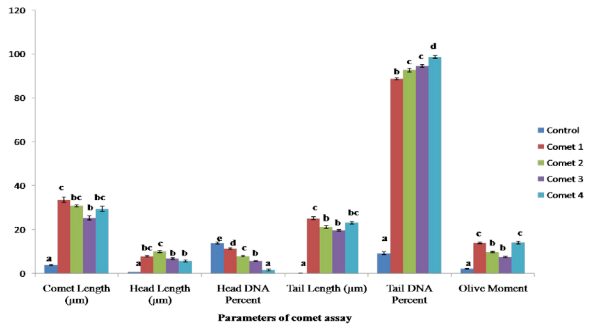

Figure 7. Comet Assay Analysis of Untreated (Control) and Lovastatin Treated HeLa Cells

prenylation of signal proteins including small GTPase (Toshihiko et al., 2010).

Apart from lovastatin other statins are also reported to be cytotoxic by inducing apoptosis in cell lines of leukemia (Dimitroulakas et al., 2000), prostate cancer (Maltese et al., 1985), colon cancer (Agarwal et al, 1999), pancreatic cancer (Muller et al.,1998; Raghunath et al., 2012). However, HeLa cells (30%) survived after 96 hours in the presence of lovastatin (1µM) (Toshihiko et al., 2010).

Hydroxy radical scavenging activity of lovastatin

Since, MTT assay gave encouraging results showing reduced proliferation of HeLa cells, further investigations were performed to know whether lovastatin also exhibits

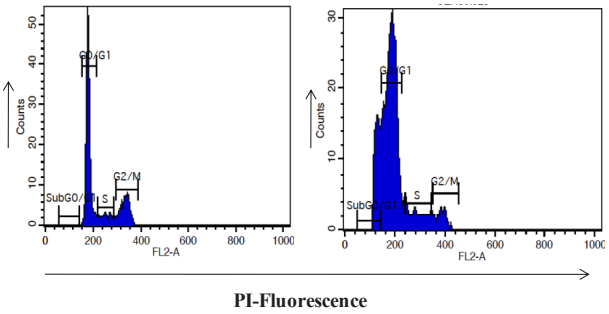

Figure 8. Flow Cytometric Analysis of HeLa Cells Treated with Lovastatin

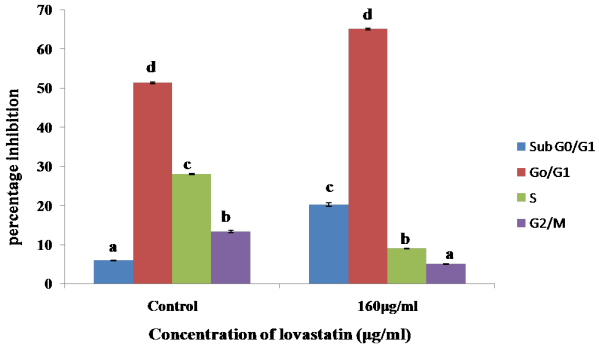

Figure 9. Cell Growth Arrest (Percentage) of HeLa Cells by Purified Lovastatin at SubG0/G1, G0/G1, S and G2/M phases

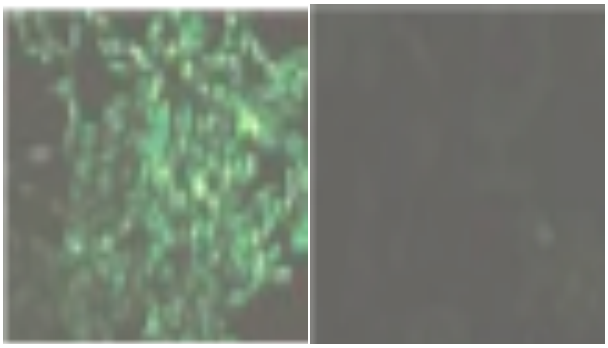

Figure 10. Fluorescence Microscopic Images of Untreated and Lovastatin Treated HeLa Cells

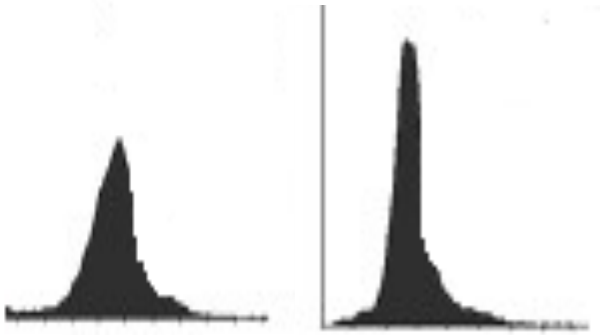

Figure 11. Flow Cytometric Analysis of Rh123 Treated HeLa Cells (Control) and Rh123 Treated HeLa Cells Exposed to Lovastatin

antioxidant property. Therefore, the radical scavenging assay and total reduced glutathione assays were carried out.

As flavonoids are known for its antioxidant property, catechin (a flavonoid) was selected as a positive control. Hydroxyl radicals are generated in the reaction mixture using ascorbic acid–iron EDTA complex. Decreased absorbance of reaction mixture signifies increased scavenging activity, as the system prevents uncontrolled formation of hydroxyl free radicals generated by cancer cells which can lead to death of healthy cells, tissues, membrane peroxidation, DNA modification, enzyme inactivation, and oxidation of proteins (Mohan kumari et al., 2011). Thus lovastatin was effective to accelerate hydroxyl radical scavenging activity (54.06%) at an IC_{50} of 3601μg/ ml as compared to its positive control (catechin) which showed 86.86% at an IC_{50} value of 350.5μg/ml (Figure 4).

There are many reports of lovastatin exhibiting antioxidant properties, inhibiting lipid peroxidation (Ajith et al., 2006), however there are very few reports of lovastatin scavenging for hydroxyl radicals. Other than lovastatin, all other statins tested, showed significant antioxidant activity against both peroxyl and hydroxyl radicals. Fluvastatin was the most active anti-oxidant among statins toward peroxyl radicals, while simvastatin was active against hydroxyl free radicals (Ferdinando et al., 2003).

Effect of lovastatin on total reduced Glutathione (GSH)

Glutathione (GSH) is a tripeptide formed by glutamic acid, cysteine, and glycine. GSH plays an important role in a many cellular processes, which include cell differentiation, proliferation, apoptosis, antioxidant defenses and progression of diseases like cancer. GSH levels are observed to increase in tumor cells, which make the neoplastic tissues resistant to chemotherapy. Hence in the current study the levels of GSH was determined to understand whether the inhibitory effects of lovastatin observed on growth of HeLa cells was also accompanied by a depletion in the levels of total reduced glutathione in the cells (Traverso, 2013).

However, in our study, lovastatin showed moderate reduction in total reduced glutathione (41.58%) as compared to its positive control (ascorbic acid) which showed 68.85% at an IC_{50} value of 19.46μg/ml (Figure 5). Thus, treatment with lovastatin could be very effective to sensitize tumor cells to cytotoxic/apoptotic effects of specific anticancer agents, by depleting GSH, thereby inhibiting GSH-mediated cell protective effect offered to the cancer cells. However, reduced GSH may induce antioxidant enzymes which could cause death of normal cells, but lovastatin prevents the exertion of oxidative stress caused by free radicals on cells by inhibiting lipid peroxidation (Mohankumari et al., 2011).

Effect of lovastatin on DNA damage and fragmentation -Comet assay

Increased DNA synthesis and cell proliferation are generally regarded as characteristic features of cancers and current therapy focuses on DNA fragmentation of these cancer cells.

Thus, result of the comet assay implicated that lovastatin showed moderate DNA fragmentation at

320μg/ml (Figure 6) and most common parameters of comet assay are analysed by opencomet software. This study was carried out to establish which measurement(s) in the comet assay are significantly correlated with DNA damage (Figure 7). The observed DNA fragmentation may be attributed to the depletion of intracellular mevalonate as observed by Choi and Jung(1999) in C6 glial cells.

Effect of lovastatin Mitochondrial Membrane Potential (MMP) of HeLa cells

This study was performed to check the effect of lovastatin through its intrinsic pathway involving the mitochondria. Hela cells treated with lovastatin at 150μg/ml, showed significant decrease in uptake (10.03%) of Rhodamine 123 (Rh123) as compared to untreated cells which showed 80%. Fluorescence microscopy and flow cytometry analysis of rhodamine treated HeLa cells are shown in Figure 10 and Figure 11 respectively.

The mitochondria of healthy cells maintain an electrochemical gradient in its inner membrane created by the movement of protons across its transmembrane space called mitochondrial membrane potential (MMP) (Lincoln et al., 1981). Deregulation of this cellular pH (MMP) of mitochondrial transmembrane of HeLa cells by lovastatin results in cell death. Reduction in MMP could be due to the opening of the mitochondrial permeability transition pores, resulting in the release of proteins and cytochrome C into the cytosol, which in turn triggers other downstream events in the apoptotic cascade leading to apoptosis of cells. (Matsuyama and Reed, 2000). Mitochondria-rhodamine specific interaction is dependent on the high MMP which is maintained by functional mitochondria. Thus uptake of rhodamine 123 (a fluorescent dye) is decreased due to reduction in MMP (Lincoln et al., 1981).

Effect of lovastatin on cell cycle arrest of HeLa cells

Further, results of all the above assays incited us to verify the cell cycle arrest pattern of lovastatin on HeLa cells. Flow cytometric analysis of lovastatin treated HeLa cells showed suppressed proliferation which was evident at 320μg/ml of lovastatin.

The cell cycle distribution in cultures exposed to a cytostatic concentration of lovastatin was unchanged during the initial 6h of treatment. However, as the time of exposure to lovastatin increased, the proportion of cells in S phase progressively decreased concomitant with an increase in G1-phase cells. Hence, lovastatin suppressed growth of Hela cells. Cells show cell cycle arrest of 20.09% and 24.67% at SubG0/G1 phase at the concentration of 160 and 320μg/ml respectively compared to the untreated cells (0.0%). However, arrest at G2/M phase of cell cycle is not much different as compared to untreated cells with above mentioned concentration (Figure 9). This result suggests that lovastatin do not allow the cells to proceed from G0/G1 to next phase of cell cycle and at the same time it might induce the apoptosis. This induced apoptosis may be due to elevation of some cell cycle inhibitors that have been reported to suppress cell cycle progression, resulting in G0/G1 phase arrest in HeLa cells (ref). It is also possible that inhibition of isoprenylation of certain proteins by lovastatin may be responsible for the suppression of cell growth (Jakobisiak et al., 1991).

Further, lovastatin is also reported to cause G1 phase arrest in Swiss 3T3 mouse fibroblast cells (Poon et al., 1995). These effects of lovastatin could be due to the induction of multiple intrinsic stress pathway in HeLa cells, which happens either by activation of certain stress response like caspases or inhibiting some growth factors (Laurie et al., 2012). Cell growth arrest may also be attributed to the down-regulation of cyclin dependent kinase (CDK 2) activity and cytostatic activity of lovastatin that could be mediated by the closed ring form of lovastatin by affecting proteasome function (Wong et al., 2002). Also, cells in the late G0 and G1/G2 phases of the cell cycle are reported to be most sensitive to cell death when exposed to ionizing radiation, whereas cells in S phase are most resistant. Thus, G1 phase cell cycle arrest, induced by lovastatin can potentially sensitize HeLa cells to radiation, making the radiation therapy more effective (shibata et al., 2004).

In conclusion, the present study adds to the understanding that lovastatin is not only capable of reducing cardiac disease related mortality but also could be a potential anticancer agent. This is in agreement with previous reports that lovastatin reduces cancer incidence worldwide by 28-33% (Julie et al., 1997; Elena et al., 2008; Linda et al., 2010). Results also suggest the probable use of lovastatin in association with conventional treatment as apoptosis-triggering agents in HeLa cells as statins demonstrate to potentiate the antitumor activity of some cytokines. However, the interaction between lovastatin and chemotherapeutic agents could be a field of considerable research interest in future. Overall, lovastatin from *A.terreus* (KM017963) gives supporting evidence as an agent for chemotherapy of cervical cancer.

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