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Research Article

CYTOTOXIC AND APOPTOTIC NATURE OF MIGRASTATIN, A SECONDARY METABOLITE FROM STREPTOMYCES EVALUATED ON HEPG2 CELL LINE

VINAYAGAM RAMBABU[A], S. SUBA[B], P. MANIKANDAN[B], SUBURAMANIYAN VIJAYAKUMAR[A*]

[a*]P.G and Research Department of Botany and Microbiology, A.V.V.M. Sri Pushpam College (Autonomous), Poondi, Thanjavur, [b]K.K Biotec Lab Service, Chennai, Tamil Nadu, India. Email: svijaya_kumar2579@rediffmail.com

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ABSTRACT

Objective: The commercially available migrastatin is known for its antitumor activities. To further establish its role in anticancer activity, we tested its potential against human hepatoma cell line (HepG2). We investigated the molecular mechanisms of migrastatin induced apoptosis and growth arrest in human hepatoma cell lines.

Methods: Upon treatment with migrastatin, a time dependent inhibition of cell growth was observed and cells developed many hallmark features of apoptosis. The cell viability was assessed by MTT assay and LDH and GSH content of the cell line were also assessed for the cytotoxic nature of the migrastatin.

Results: Light microscopic study reveals the cell clumping and apoptotic body formations. Migrastatin induced growth inhibition was associated with induction of p53 and activation of caspase-3.

Conclusion: Thus, we bring to a close that migrastatin induces cell death and apoptosis in HepG2 cell line.

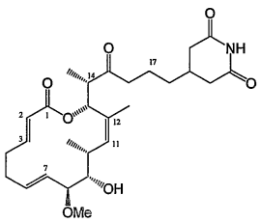
Keywords: Migrastatin, Actinomycetes, Streptomyces, Apoptosis, Secondary metabolite

INTRODUCTION

Natural products have demonstrated to be precious resource for the discovery of new drug candidates, and also as tools for chemical biology and medicinal chemistry research. Numerous natural products have also long been used to treat various human disorders and distinguished by their drug like possessions [1,2]. Microbial natural products are an important source of both existing and new drugs [3]. Among the producers of commercially important metabolites, actinomycetes have proven to be a prolific source with a surprisingly small group of taxa accounting for the vast majority of compounds. *Actinomycetes* are the most economically and biotechnologically valuable prokaryotes. They are widely distributed in natural and manmade environments play an important role of the microbial community, responsible for degradation and recycling of natural biopolymers, such as cellulose, lignin and chitin [4]. It is also a source of a wide range of other types of bioactive compounds for biotechnological applications [5]. *Actinomycetes* are non-motile, filamentous, Gram-positive bacteria. *Streptomyces*, the largest genus of Actinobacteria, is a group of Gram-positive and generally high GC-content bacteria which produce spores from aerial filaments called sporophores. These rise above the colony and form spores called conidia by simple cross-wall divisions of the filament [6-9]. The species belonging to the genus *Streptomyces* are well known for producing a variety of bioactive secondary metabolites including antibiotics, immunomodulators, anticancer & antiviral drugs, herbicides, and insecticides [10].

Secondary metabolites produced by actinomycetes acquire a wide range of biological activities [11,12] and the vast majority of these compounds are derived from the single genus *Streptomyces* [13]. *Streptomyces* species are distributed widely in marine habitats and are of commercial interest due to their unique capacity to produce novel metabolites. *Streptomyces* species are found worldwide in soil and are important in soil ecology. Much of the characteristic earthy smell of soils arises from chemicals called geosmin given off by *Streptomyces species*. Unlike most bacteria, *Streptomycetes* possess linear chromosomes [14]. The biochemistry of *Streptomycetes* is truly remarkable, considering their production of secondary metabolites, many of which account for almost half of all known Antibiotics [15]. Many of these compounds have important applications in human medicine as antibacterial, antitumour and antifungal agents. Also, in agriculture these compounds act as growth promoters, agents for plant protection, antiparasitic agents and herbicides [16].

Development of new therapeutics preventing tumour metastasis is urgently needed, since tumour metastasis is the primary cause of death of cancer patients. Migrastatin is an organic compound which naturally occurs in the *Streptomyces* have shown to have potential in treating cancer, are potent inhibitors of metastatic tumour cell migration, invasion and metastasis [17].



Structure of Migrastatin

In this framework, the present investigation endeavor to appraise the anticancer property of migrastatin, secondary metabolite of *Streptomyces platensis* bacteria against human hepatoma cell line HepG2 - *in vitro* study.

MATERIALS AND METHODS

Drug and Chemicals

Dimethyl sulfoxide (DMSO) was purchased from sigma, St Louis, MO, USA. Migrastatin was purchased from MedKoo Biosciences,USA. RPMI-1640 and sodium pyruvate were purchased from Biochrom, Berlin, Germany. Penicillin-streptomycin and fetal bovine serum were purchased from Gibco, Germany. Trypsin-EDTA was obtained from Himedia Laboratories Pvt Ltd, Mumbai. Cell culture plates and dishes were purchased from TPP, Switzerland. Primary antibodies such as p53 and Caspase-3 were purchased from Abcam Laboratories, USA and Novocastra Laboratories Ltd, Newcastle, UK, respectively. Rabbit anti-mouse IgG was purchased from Bangalore Genei, India. Nitrocellulose membrane was obtained from Millipore, Bedford, USA. All other chemicals including solvents were of highest purity and of analytical grade marketed by Glaxo Laboratories and SISCO Research Laboratories (SRL), Mumbai, India.

Drug Preparation

Migrastatin was dissolved in Dimethyl sulfoxide (DMSO) (final concentration of the DMSO was not exceeded 0.1% (v/v) and did not affect the cell survival) prepared in serum free RPMI medium and filtered by 0.045 mm syringe filter and stored at 4ºC.

Cell Culture and Harvesting of HepG2

Human hepatoma cell line (HepG2) was obtained from National Center for Cell Sciences (NCCS), Department of Biotechnology, Pune, India. Cells were grown as monolayer in RPMI-1640 medium, supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS), antibiotics (Penicillin 100 U/ml, Streptomycin 10 µg/ml) and 1mM sodium pyruvate under standard conditions (37ºC, 5% CO_2) in a controlled humidified atmosphere. The medium was changed every three days. Cultured cells were starred, for 24 h trypsinized (0.05% trypsin and 0.02% EDTA), seeded at a density of 1 x 10^4 cells per well in 96 well plate for MTT assay or at a density of 0.5x10^6 cells per well for LDH leakage assay, GSH assay, western blot analysis and incubated with or without 6µM or 10µM migrastatin for 24 h. After the treatment, cells were trypsinized and centrifuged at 2500 rpm for min.

***In vitro* assay**

Cell proliferation assay by 3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method was performed [18]. Lactate dehydrogenase (LDH) leakage assay was performed [19]. Total reduced glutathione (GSH) was determined by the method [20].

Morphologiacl Assessment of Apoptosis by Light Microscopic Studies

Light microscopic examination of the cells was performed to observe the morphological changes after the treatment with migrastatin for 24 h. The HepG2 cells were grown in 100 mm sterile petriplates and treated with migrastatin at the concentrations of 6 and 10µM. Cells were then fixed for 5 min with 10% methanol and morphological changes were observed under inverted microscope (Nikon, Japan).

Expression of p53 and caspase - 3 proteins by Western blotting

HepG2 cells (1x10^6/ml) were treated with the migrastatin at the concentration of 6 µM and 10 µM for 48 h at 37°C. Cells were lysed with 10 µl of lysis buffer. Cell proteins were separated in a sodium dodecyl sulfate polyacrylamide gel elecrophorosis (SDS-PAGE). 4% stacking gel and 10% resolving gel were used to separate the proteins. After electrophoresis, gel was placed over a nitrocellulose membrane, separate blotting was done for each protein. The gel and the PVDF membrane were packed by three cut-pieces of whatmann filter paper (No.3). This set up was covered on both sides with absorbers and clipped. The whole set up was immersed in a tank containing blotting buffer. A current of 25 mA was passed through overnight.

Then, the membrane was removed from the system and immersed in methanol for a minute. The membranes were blocked by treating with the blocking buffer for 1 h at 37°C. After washing, the membranes were incubated with anti-mouse p53 (1:100) and anti-mouse caspase - 3 (1:1000) for 6 h at 37°C. After three washes in PBS / 0.1% Tween 20, the membrane was incubated with Horse Raddish Peroxidase (HRP) conjugated anti-mouse IgG antibody for 1 h at 37°C. The bands developed were then visualizes and photographed. The band intensity for P53 and caspase 3 was normalized with that of the internal control β actin.

RESULT

Recently the cytotoxic effects of various chemicals and natural substances on malignant tumor cells in culture have been extensively studied as a primary screening for anti tumor activities [21]. Figure 1 illustrates the cell viability of control and migrastatin treated (2µM, 4µM, 6µM, 8µM and l0 µM) HepG2 cells. In the present study, the migrasatin conspicuously inhibited the HepG2 cells at the concentration of 6 µM and l0 µM after 24 h and 48 h of treatment. The results showed that treatment with migrastatin markedly reduced the viability of HepG2 cells in a dose dependent manner.

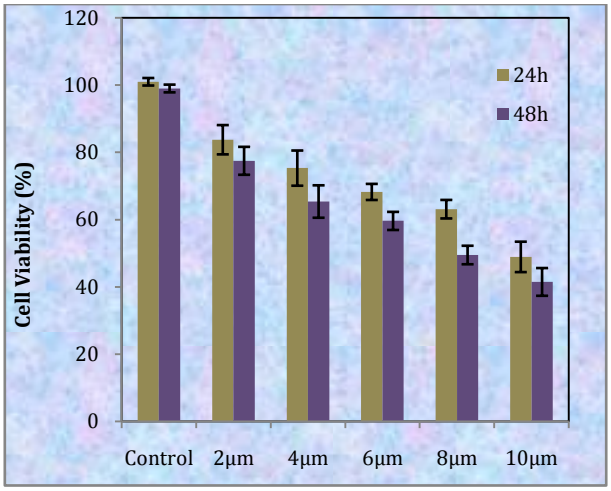


Fig. 1: Effect of various concentration of Migrastatin on HepG2 cell lines - MTT ASSAY

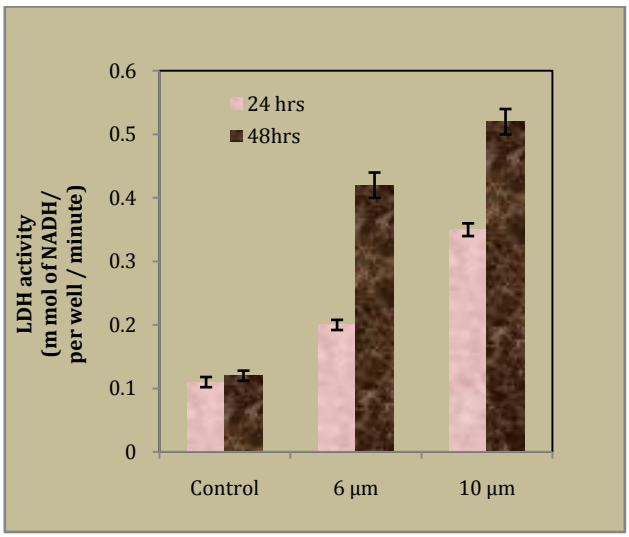


Fig. 2: LDH activity in HepG2 cell line treated with Migrastatin

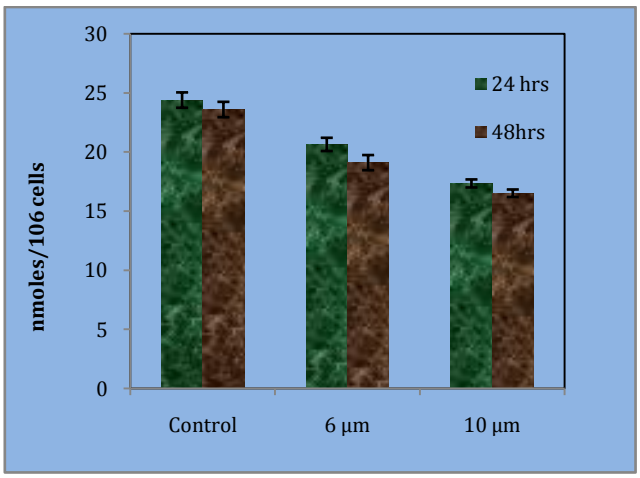
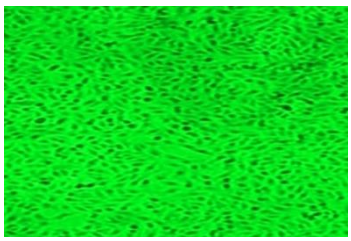


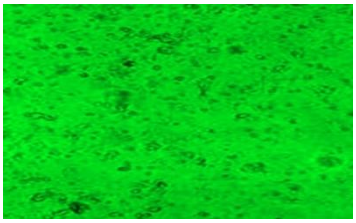
Fig. 3: GSH activity in HepG2 cell line treated with Migrastatin

The levels of lactate dehydrogenase (LDH) released into the medium of control and migrastatin treated (6 µM and l0 µM) HepG2 cells were presented in Figure 2. From this result it was observed that LDH activities found to be significantly elevated after 48 h of exposure in the medium containing migrastatin when compared to the control. It was well known that the toxicity of anti-tumor drugs may largely depend on the intracellular level or reduced glutathione

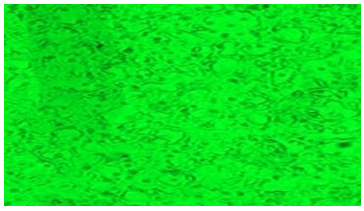
(GSH) [22]. Glutathione plays an important role in protecting cells and cellular components against oxidative stress and in detoxification. It was often found that GSH levels were increased in the drug resistant cancer cells when compared to the drug sensitive cells. Inhibition of glutathione synthesis or modulation of glutathione storages in tumors to reduce anticancer drugs resistance may contain a novel anticancer strategy [23]. The levels of GSH content in control and migrastatin treated HepG2 Cells were presented in figure 3. The significant depletion of GSH was observed in treated HepG2 cells at the concentration of 6 µM and 10 µM/ml compared to the control cells. The morphological changes of control and migrastatin treated HepG2 cells at the concentration of 6 and 10 µM for 24 h of exposure displayed in figure 4. Control HepG2 showed swelling and rounded morphology of the cells with condensed chromatin and their membrane. This may lead to crooked and vesicle shaped. On the contrary, migrastatin treated HepG2 cells, monolayer obliteration was distinguished. Progressive structural alterations and reduction of HepG2 cell populations were observed in both the concentrations.



Control



Migrastatin 6µM

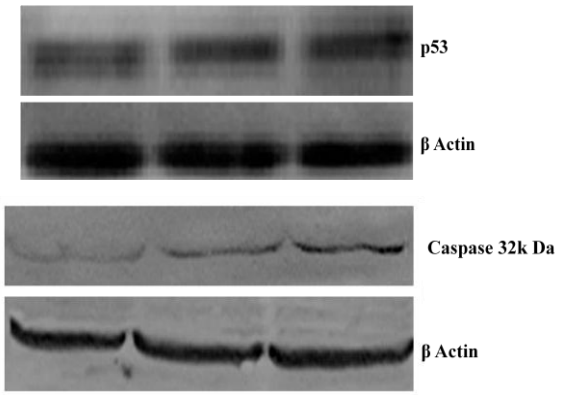


Migrastatin 10µM

Fig. 4: Light Microscopic studies of control and Migrastatin treated HepG2 cells

A lot of oncogenes and tumor suppressor genes were involved in mediating apoptosis. The p53 gene which was strongly implicated in animal and human carcinogenesis and it was a significant regulator of the process of apoptosis [24]. After DNA damage in cell types, p53 can trigger the genetically altered cells to be eliminated by inducing apoptosis [25]. In addition to its DNA damage response, p53 was also involved in the response by abnormal or stress conditions such as hypoxia, Oxidative stress, the presence of genotoxic chemicals and depletion of ribonucleotides, [26]. Therefore normal p53 expression and function were crucial to prevent the propagation of genetically damaged cells and to prevent proliferation of cells under stress conditions. It was well known that after initiation of the apoptotic program, the release of cytochrome-C from mitochondria triggers the activation of caspase-3 and the consequent rapid cleavage of Poly (ADP-ribose) polymerase (PARP), a substrate of caspase 3 [27].

The expression of p53 and caspase-3 protein in control and migrastatin treated (6 µM and 10 µM) HepG2 cells by western blotting was presented in figure 5. Administration of migrastatin increase the band intensity of 53 kDa protein compared to the control. The accumulation of p53 protein indicates the expression of tumor suppressor protein induced apoptosis in migrastatin (6 µM and 10 µM) treated HepG2 cells. From the result of our present investigation, it was observed that treatment of HepG2 cells with migrastatin increased intensity of 32 kDa band and appearance of low molecular weight protein below the 32 kDa in 6 and 10 µM treated HepG2 cells. This result strongly suggests that drug treatment stimulated the proteolytic cleavage of caspase-3 protein and initiate the apoptosis.



Lane 1 – Control

Lane 2 – 6 µM Migrastatin

Lane 3 – 10 µM Migrastatin

Fig. 5: Western Blotting analysis of p53 and Caspase-3 protein expression in control and Migrastatin treated HepG2 cells

DISCUSSION

HepG2 cells, a human hepatoma cell line, are considered to be a good model for studying in vitro xenobiotic metabolism and toxicity to the liver since they retain many of the specialized functions which characterize normal human hepatocytes [28]. Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals. The choice of using a fastidious viability or cytotoxicity assay technology may be influenced by specific research goals. Cytotoxicity frequently has a broad and imprecise importance in the drug discovery. For *in vitro* cell culture systems, a compound or treatment is considered to be cytotoxic if it interferes with cellular attachment, significantly alters morphology, adversely affects cell growth rate, or causes cell death [29]. MTT assay is the most common employed for detection of cytotoxicity or cell viability following exposure to toxic substances. It is widely used to measure cell proliferation and for screening of anticancer drugs [30]. It is based on the reduction of tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) by actively growing cells to produce a purple formazan product. MTT is a water soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells [31]. From the results it is inferred that the exposure of different concentration of migrastatin (6 and 10 µM/ml) for 24 h resulted decrease of cell proliferation in a dose dependent manner. Thus the inhibitory effect on HepG2 cells by migrastatin strongly proves the anti-proliferation possessions of secondary metabolite migrastatin.

LDH has long been favored as a marker of cell death for *in vitro* models [32]. Lactate dehydrogenase (LDH) cellular enzyme is used as cell death markers. However, other celluler enzymes such as adenylate kinase and glucose-6-phosphate are not stable and only lactate dehydrogenase does not lose its activity during cell death assays [33]. Therefore, cell death assays based on LDH activity are more reliable than other enzyme-based cell death assays. The LDH leakage assay is based on the measurement of lactate

dehydrogenase activity in the extracellular medium. Reliability, speed and simple evaluation are some of the characteristics of this assay. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage [34**].** LDH is a more reliable and more accurate marker to study the cytotoxicity. Because damaged cells are fragmented completely during the course of prolonged incubation with toxic substances [35]. In the present study, the lactate dehydrogenase leakage was elevated drastically in migrastatin treated HepG2 cells when compared with the control HepG2 cells. The result of our present investigation put forward that the inhibition may be due to the anticarcinogenic potential of migrastatin. Consequently, it was recommended that the lactate dehydrogenase leakage in HepG2 cells may be due to cytotoxic nature of secondary metabolite and authenticate its antitumour property.

The intracellular oxidative metabolites play a significant role in the regulation of apoptosis. For instance, some apoptosis inducing agents are either oxidant of cellular oxidative metabolites. Therefore, the measurement of GSH in drug treated HepG2 cells constitutes the supporting evidence for apoptosis. Glutathione (GSH) is a tripeptide contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain. Glutathione, an antioxidant which protect cells from reactive oxygen species such as free radicals and peroxides [36]. Glutathione is nucleophilic at sulphur and attacks poisonous electrophilic conjugate acceptors. GSH is found almost exclusively in its reduced form, since the enzyme that reverts it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress. The proportion of reduced glutathione to oxidized glutathione within cells is used scientifically as a measure of cellular toxicity [37]. Reduced glutathione has been hypothesized to participate a responsibility in the rescue of cells from apoptosis, by buffering an endogenously induced oxidative stress [38].

It was reported that *Salvia miliorrhiza* inhibited human hepatoma HepG2 cells growth and induced apoptosis involving intracellular GSH depletion [39]. Onset of apoptosis was associated with a fall of intracellular GSH in different cellular system [40]. Loss of GSH was shown to be tightly coupled with a number of downstream events in apoptosis [41]. In the present examination, it was observed that the levels of GSH were significantly decreased in migrastatin treated HepG2 cells at the concentration of 6 and 10 μM. This indicates that the decrease in GSH may be involved in the inhibition of HepG2 cells growth and cause apoptosis.

The present study point toward that, the migrastatin might quickly encourage intracellular oxidation in HepG2 cells and cause apoptosis which lead to cell death. Therefore, the cytotoxic action of this migrastatin may be attributed to its pro-oxidant action on the cells.

In the present analysis, observation of migrastatin in light microscopic studies drug treated HepG2 cells at concentrations of 6 and 10μM after 24 h of exposure showed the characteristic morphological features of apoptosis in HepG2 cells. The morphological alterations observed were diminution in cell volume, cell shrinkage, reduction in chromatin condensation and formation of cytoplasmic blebs. However, the control HepG2 cells were seen with higher confluence of monolayer without any devastation.

Apoptosis is an energy-dependent, tightly regulated and selective physiological process that governs the removal of supernumerary or defective cells. It occurs under normal physiological condition. It can also be triggered by diverse pathologies. In healthy tissues, the main role of apoptosis is to maintain optimal number of cells in tissues and organs by removing the redundant, damaged or functionally abnormal cells [42]. A lot of oncogenes and tumor suppressor genes were involved in mediating apoptosis. The p53 gene which was strongly implicated in animal and human carcinogenesis and it was a significant regulator of the process of apoptosis [43]. Mutation of p53, a classical tumor suppressor, is frequently associated with

oncogenesis. Cellular functions modulated by the p53 protein include DNA synthesis, DNA repair, cell cycle arrest, gene transcription, senescence and apoptosis [44]. The tumor suppressor p53 is a key regulator of cell cycle arrest and apoptosis. Its activity and stability are regulated by a complex network of post-translational modifications and molecular interactions influenced by several signaling pathways A mutation in the p53 gene causes p53 inactivation in 50% of human cancers [45]. p53 is an extremely efficient inhibitor of cell growth, inducing cell cycle arrest and/or apoptotic cell death, depending on cell type and environment. Therefore, regulation of p53 activity is critical to allow normal cell division [46].

Therefore normal p53 expression and function were crucial to prevent the propagation of genetically damaged cells and to prevent proliferation of cells under stress conditions. Tumor suppressor gene p53 is one of the critical genes regulating the onset of DNA replication around G1/S boundary. Also, p53 mediated tumor suppression appears to be critical for therapeutic potential in treatment of tumors [47] p53 contributes to apoptosis induced by a variety of cellular stresses, including DNA damage, oxidative stress and chemotherapeutic drugs [48]. In the present study, migrastatin treated HepG2 cells showed upstream regulation of p53 protein expression after exposed to the concentrations of 6 μM and 10 μM for 48 h. Thus it is inferred that, migrastatin may possibly enhance the susceptibility of HepG2 cells to apoptosis by attenuating the tumor suppressor protein. In control cells, as evident from the low levels of p53 at both gene and protein level, it was speculated that mdm2 might bind to the p53 just to evacuate it from nucleus to cytosol.

Caspases are synthesized as proenzymes that contain an active site of cystein nucleophile [49] which is prone to oxidation or thiol alkylation [50]. Cleavage at specific aspartate residues converts the proenzymes into biologically active cysteine proteases. The activated caspases abrogate the effect of substrates that protect cellular integrity, such as the DNA-repair enzyme poly(ADP-ribose) polymerase (PARP), and thereby induce apoptotic cell death. The activation of at least one caspase appears to be an essential step in cellular apoptosis [51, 52]. Caspase-3 is a member of the Interleukin-1β Converting Enzyme (ICE) family of cysteine proteases. Caspase-3 exists in cells as an inactive 32 kDa proenzyme, called pro-Caspase-3. The overexpression of Caspase-3 can result in apoptosis. Likewise, the inhibition of Caspase-3 or other caspases can prevent cells from entering the apoptotic pathway [53].

Caspase 3, the final executor was in high active in the migrastatin treated groups which was evident from the increased expression of protein in western blot analysis. Caspase-3 may then cleave vital cellular proteins or activate additional caspases by proteolytic cleavage. In the present investigation, migrastatin treated HepG2 cells showed a low intensity of 32-kD protein band and 17- kD protein band. Caspase-3 usually exits as an inactive pro-caspase 3 that becomes proteolyically activated by multiple cleavages of its 32-kD precursor to generate the 20/11 or 17/11-kD active forms in cells undergoing apoptosis [54]. Thus, the appearance of 17- kD protein may be one of the active forms of caspase-3 protein. It may be due to proteolytic cleavage of inactive caspase-3 induced by secondary metabolite migrastatin. This occurs before the further activation of caspase-3-mediated apoptosis. Recent studies have also suggested that the proteolytic degradation of specific substrates is responsible for many of the morphological features of apoptosis [55].

CONCLUSION

Migrastatin,a secondary metabolite from *Streptomyces* was evaluated on HepG2 cell line. From MTT assay, the cell viability was assessed and LDH and GSH content of the cell line were also tested for to assess the cytotoxic nature of migrastatin. From the result it could be concluded that migrastatin has potent cytotoxicity on HepG2 cells on time and dose dependent manner. Cell clumping and apoptotic body formations were observed by Light microscopic study. In addition, Migrastatin could capable of inducing apoptosis through the expression of p53 and caspase 3.Thus, migrastatin has potent cytotoxic and apoptotic nature on HepG2 hepatoma cell line.

REFERENCES

1. Solanki R, Khanna M, Lal R. Bioactive compounds from marine actinomycetes. Indian J Microbiol 2008; 48: 410–431.
2. Wu SJ, Fotso S, Li F, Qin S, Laatsch, H. Amorphane Sesquiterpenes from a Marine *Streptomyces sp.* J Nat Prod 2007; 70: 304–306.
3. Williams PG, Asolkar RN, Kondratyuk T, Pezzuto JM, Jensen PR, Fenical W. Saliniketals A and B, Bicyclic Polyketides from the Marine Actinomycete Salinispora arenicola. J Nat Prod 2006; 70: 83–88.
4. Semêdo LT, Linhares AA, Gomes RC, Manfio GP, Alviano CS, Linhares LF, Coelho RR. Isolation and characterization of actinomycetes from Brazilian tropical soils. Microbiol Res 2001; 155(4): 291-9.
5. Bull AT, Goodfellow M, Slater JH. Biodiversity as a source of innovation in biotechnology. Annu Rev Microbiol 1992; 46: 219-52.
6. Li F, Maskey RP, Qin S, Sattler I, Fiebig HH, Maier A, Zeeck A, Laatsch H. Chinikomycins A and B: Isolation, structure elucidation, and biological activity of novel antibiotics from a marine *Streptomyces sp.* Isolate M045. J Nat Prod 2005; 68: 349–353.
7. Maskey RP, Li FC, Song Q, Fiebig HH, Laatsch H. Chandrananimycins A–C: Production of novel anticancer antibiotics from a marine *Actinomadura sp.* Isolate M048 by variation of medium composition and growth conditions. J Antibiot 2003; 56: 622–629.
8. Wu SJ, Fotso S, Li F, Qin S, Kelter G, Fiebig HH, Laatsch H. N-carboxamido-staurosporine and selina-4(14),7(11)-diene-8,9-diol, new metabolites from a marine *Streptomyces sp.* J Antibiot 2006; 59: 331–337.
9. Ding L, Pfoh R, Ruhl S, Qin S, Laatsch H. T-Muurolol sesquiterpenes from the marine *Streptomyces sp.* M491 and revision of the configuration of previously reported amorphanes. J Nat Prod 2008; 72: 99–101.
10. Rahman MA, Islam MZ, Khondkar P, Islam MA. Characterization and antimicrobial activities of a polypeptide antibiotic isolated froma new strain of *Streptomyces parvulus*. Bangladesh Pharm J 2010; 13: 14-16.
11. Sujatha P, Bapi Raju KVVSN, Ramana T. Studies on a new marine streptomycete BT-408 producing polyketide antibiotic SBR-22 effective against methicillin resistant taphylococcus aureus. Microbiol Res 2005; 160: 119–126.
12. Miller ED, Kauffman CA, Jensen PR, Fenical W. Piperazimycins: Cytotoxic Hexadepsipeptides from a Marine-Derived Bacterium of the Genus *Streptomyces*. J Org Chem 2006; 72: 323–330.
13. Moore BS, Trischman JA, Seng D, Kho D, Jensen PR, Fenical W. Salinamides, antiinflammatory depsipeptides from a marine streptomycete. J Org Chem 1999; 64: 1145–1150.
14. Dharmaraj S. Marine *Streptomyces* as a novel source of bioactive substances. World J Microbiol Biotechnol 2010; 26: 2123–2139.
15. Berdy J, Horvath I, Szentirmai A. Antibiotics produced by Streptomyces. II. the tautomeric transformation of xanthomycin. Z Allg Mikrobiol. 1964; 61: 232-5.
16. Hopwood DA, Chater KF, Bibb MJ. Genetics of antibiotic production in Streptomyces coelicolor A3(2), a model streptomycete. Biotechnology 1995; 28: 65-102.
17. Shan D, Chen L, Njardarson JT, Gaul C, Ma X, Danishefsky SJ, Huang XY. Synthetic analogues of migrastatin that inhibit mammary tumor metastasis in mice. Proc Natl Acad Sci USA 2005; 102(10): 3772–3776.
18. Cardile V, Renis M, Scifo C, Lombardo L, Gulino R, Mancari B, Panico A. Behaviour of the new asbestos amphibole fluorendenitein different lung cell systems. Int J Biochem Cell Biol 2004; 36: 849–60.
19. Grevell AR, Berry MN. The effect of phosphate and substrate free incubation conditions on glycolysis in Ehrich ascites tumour cells. Biochim Biophys Acta 1996; 1291(1): 83–8.
20. Moron MS, Depierre JW, Mannervik B. Levels of glutathione reductase and glutathione-S-transferase activities in rat lung and liver. Biochim Biophys Acta 1979; 582: 67–78.
21. Morita K, Arimochi H, Ohnishi Y. In vitro cytotoxicity of 4-methyl catechol in murine tumour cells: induction of apoptotic cell death by extra cellular pro-oxidant action. J Pharmacol Exp Ther 2003; 306: 317-323.
22. Troyano A, Fernandez C, Sancho P, de Bias E, Aller P. Effect of glutathione depletion on anti-tumour drug toxicity (apoptosis and necrosis) in U-9371 promonocytic cells. The role of intracellular oxidation. Biol Chem 2001; 276: 107-15.
23. Rudin CM, Yang Z, Schumaker LM, Vander Weele DJ, Newkirk K, Egorin MJ, Zuhowski EG, Cullen KJ. Inhibition of glutathione synthesis reverses Bc1-2-mediated cisplatine resistance. Cancer Res 2003; 63: 312-8.
24. Muller M, Strand S, Hug H, Heinemann EM, Walczak H, Hofmann WJ, Stremmel W, Krammer PH, Galle PR. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53, Journal of Clinical Investigation 1997; 99: 403-13.
25. Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993; 74(6): 957-67.
26. Linke SP, Clarkin KC, Di Leonardo A, Tsou A, Wahl GM. A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. Genes Dev 1996; 10(8): 934-47.
27. Emanuele S, Calvaruso G, Lauricella M, Giuliano M, Bellavia G, D'Anneo A, Vento R, Tesoriere G. Apoptosis induced in hepatoblastoma HepG2 cells by the proteasome inhibitor MG132 is associated with hydrogen peroxide production, expression of Bcl-XS and activation of caspase-3. Int J Oncol 2002; 21(4): 857-65.
28. Laohavechvanich P, Muangnoi C, Butryee C, Kriengsinyos W. Protective effect of makrut lime leaf (Citrus hystrix) in HepG2 cells: Implications for oxidative stress. Science Asia 2010; 36: 112–117.
29. Horvath S. Cytotoxicity of drugs and diverse chemical agents to cell cultures. Toxicology 1980; 16: 59-66.
30. Maioli E, Torricelli C, Fortino V, Carlucci F, Tommassini V, Pacini A. Critical appraisal of the MTT assay in the presence of rottlerin and uncouplers. Biol Proced Online 2009; 11: 227-240.
31. Fotakis G, Timbrell JA. *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicology Letters 2006; 160: 171–177.
32. Decker T, Lohmann-Matthes M. A quick and simple method for the quantification of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods 1988; 115: 61 -9.
33. Fukumoto M, Kujiraoka T, Hara M, Shibasaki T, Tatsuo H, Yoshida M. Effect of cadmium on gap junctional intercellular communication in primary cultures of rat renal proximal tubular cells. Life Sci 2001; 69: 247–254.
34. Korzeniewski C, Callewaert D. An enzyme-release assay for natural cytotoxicity. J Immunol Methods 1983; 64: 211-24.
35. Langeswaran K, Gowthamkumar S, Vijayaprakash S, Revathy R, Balasubramanian MP. Influence of role on Wnt signaling molecule in HepG2 cell line. J Nat Sci Biol Med 2013; 4(1): 126-133.
36. Pombella A, Visvikis A, Paolicchi A, De Tata V, Casini AF. The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol 2003; 66(8): 1499-503.
37. Pastore A, Piemonte F, Locatelli M, Lo Russo A, Gaeta LM, Tozzi G, Federici G. Determination of blood total, reduced and oxidized glutathione in pediatric subjects. Clin Chem 2001; 47(8): 1467-1469.
38. Ghibelli L, Coppola S, Rotilio G, Lafavia E, Maresca V, Ciriolo MR. Non-oxidative loss of glutathione in apoptosis via GSH extrusion. Biochem Biophys Res Commun 1995; 216(1): 313-20.
39. Liu J, Shen HM, Ong CN. Salvia miltiorrhiza inhibits cell growth and induces apoptosis in human hepatoma HepG2 cells. Cancer Lett 1999; 153: 85-93.
40. Hall AG. The role of glutathione in the regulation of apoptosis. Eur J Clin Invest 1999; 29: 238-245.
41. Dobbelsteen VDDJ, Nobel CS, Schlegel J, Cotgreave IA, Orrenius S, Slater AF. Rapid and specific efflux of reduced glutathione

during apoptosis induced by anti-Fas/APO-1 antibody. J Biol Chem 1996; 271: 15420-15427.

42. Aleksei P, Micheal Z, Tadeusz JK, Marek L. Capsases and cancer: mechanism of inactivation and new treatment modalities. Exp Oncol 2004; 26(2): 82-97.

43. Gowtham Kumar S, Ramakrishnan V, Madhusudhanan N, Thiyagarajan G, Langeswaran K, Balasubramanian MP. Apoptosis induced in Hepatoma cells (HepG2) by Allyl isothiocyanate associated with expression of P53 and activation of caspase-3. Int J Biol Pharma Res 2011; 2(1): 19-26.
44. Schumacher B, Hofmann K, Boulton S, Gartner A. The C. elegans homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis. Curr Biol 2001; 11: 1722–7.
45. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer 2002; 2: 594–604.
46. Vousden KH. Activation of the p53 tumor suppressor protein. Biochim Biophys Acta 2002; 1602: 47-59.
47. Ryan KM, Phillips AC, Vousden KH. Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol 2001; 13: 332-337.
48. Steele RJ. Thompson AM, Hall PA, Lane DP. The p53 tumor suppressor gene. Br J Surg 1998; 85: 1460-1467.
49. Nicholson DW, Ali A, Thornberry NA, Vaillancourt IP. The activity of caspases is optimal under reducing environments. Nature J 1995; 376: 37-43.
50. Samantha S, Nalinie W, Ira T, Neelakanthi R, Mayuri G. Protection against diethylnitrosamine induced hepatocarcinogenesis by an indigenous medicine comprised of Nigella sativa. J Carcenogenesis 2003; 2: 6-21.
51. Martin S, Green D. Protease activation during apoptosis: Death by thousand cuts? (review). Cell 1995; 82: 349.
52. Kumar S, Harvey NL. Role of multiple cellular proteases in the execution of programmed cell death. FEBS Lett 1995; 375(3): 169-73.
53. Jaeschke H, Fisher MA, Lawson JA, Simmons CA, Farhood A, Jones DA. Activation of Caspase 3 (CPP32)-like proteases is essential for TNF-α-induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. J Immunol 1998; 160(7): 3480-6.
54. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999; 15: 269-290.
55. Cecconi F.Apaf1 and the apoptotic machinery. Cell Death Differ 1999; 6(11): 1087-98.