Volume 64, Issue 2, 2020

Journal of Scientific Research

Institute of Science,

Banaras Hindu University, Varanasi, India.

Cytotoxicity and Induction of Apoptosis in Melanoma (MDA-MB-435S) Cells by Emodin

Saurabh Yashaswee and Surendra Kumar Trigun*

Biochemistry Section, Centre of Advanced Study in Zoology, Institute of Science, Banaras Hindu University, Varanasi – 221005.
yash117.bhu@gmail.com, sktrigun@gmail.com*

Abstract: **Melanoma is a common cancer of epithelial cell origin which exists in multiple phenotypic forms of skin cancer. Despite several advances in development of new treatment strategies, identification of a cellular target based compound with minimum side effects remains to be explored. In this respect, characterization of plant products, as anti cancer agent, appear to be a viable alternative, however, such compounds need to be screened for their cytotoxic potential against relevant cell lines *in vitro*. This paper describes the effects of Emodin, an anthraquinone obtained from roots and rhizomes of the polygonaceae plants, against a number of cell lines. As MDA-MB-435S cells (melanoma) showed lowest IC_{50} values amongst all the screened out cells, these MDA-MB-435S cells were further investigated for monitoring whether these cells undergo apoptosis due to emodin treatment. The Ethidium Bromide/Acridine Orange (EtBr/AO) staining and comet assay showed significantly enhanced number of cells undergoing apoptosis due to emodin treatment. This was consistent with the enhanced release of M4 Lactate dehydrogenase (LDH), a known biochemical marker of cell death *in vitro*. The findings suggest that emodin causes cytotoxicity against MDA-MB-435S cells by inducing apoptosis.**

Index Terms: **Apoptosis, Cytotoxicity, Emodin, LDH, MDA-MB-435S.**

I. INTRODUCTION

Malignancies of epithelial cell origin account for 80 - 90 percent of the total cancer burden. The patients with melanoma, a common form of skin cancer, show survival rate of less than 5% and thus invites special attention for searching therapeutic strategy on priority (Homsi et al., 2005; Ali et al., 2013; Howlader et al., 2019). The melanoma incidence is age and sex specific. It usually affects women around/after 40 years and men of around 50 years age group (Rastrelli et al., 2014). Out of the current therapeutic options employed, like; surgery, chemotherapy, radiotherapy, photodynamic therapy and immunotherapy, chemotherapy is one of the most preferred clinical choice, however, inherently carries the limitation of serious side effects. To make the therapy physiology friendly, natural plant derived factors hold a great promise, however, need to be characterized with respect to their specificity and cellular targets. The advancements made in this respect during recent past suggest that the most widely used plant derived compounds for cancer chemotherapy belong to the families of quinones (Simple quinones and anthraquinone derivatives), polyphenols and xanthenes.

II. LITERATURE SURVEY

Anthraquinones belong to the aromatic compound groups with a core structure containing 9, 10- dioxoanthracene (Chien et al., 2015). Broadly these are derivatives from rhubarb; chrysophal, emodin, aloe emodin and rehin. Emodin (1, 3, 8 – trihyrdoxy-6-methylanthraquinones) is an anthraquinone derivative commonly obtained from the laxative herbs like; *Rheum palmatum* (Wang et al., 2017), *Polygonum cuspidatum* (Zhao et al., 2013), *Polygonum multiflorum* (Liu et al., 2018) and *Cassia obtusifolia* (Yang et al., 2003). This compound has been reported to show pleiotropic effects (Monisha et al., 2016; Deitersen et al., 2019) and posses a broad range of activities like; anti – inflammatory (Chen et al., 2015), anticancer, anti microbial and neuroprotective (Dong et al., 2016).

Pharmacokinetics of emodin depends on route of administration. For example, LD_{50} of 35 mg/kg body weight could be observed when injected intraperitoneally however, no adverse effect has been recorded at oral dose of even 50 – 60 mg/kg for 14 weeks. For the rodents, the lowest observed adverse effect level (LOAEL) has been reported around 6000 ppm in mice (Natl Toxicol Program Tech Rep Ser., 2001). The anti-inflammatory and ROS scavenging properties of emodin is conferred due to the presence of a hydroxyl group at 1st and 8th position of this compound (Srinivas et al., 2007). Some studies

* Corresponding Author

suggest its primary role as a cell cycle inhibitor (Chen et al., 2010) and reported to regress lung carcinoma growth (Su et al., 2005 & 2017; Tang et al., 2017), Hepatocellular carcinoma (Cui et al., 2016; Lin et al., 2016; Zhang et al., 2017; Dong et al., 2018; Zhou et al., 2019), Gastric cancer (Chihara et al., 2015), colon carcinoma (Xie et al., 2014; Liu et al., 2015; Ma et al., 2018; Wang et al., 2018;), Squamous Carcinoma of oral cavity (Zhang et al., 2015; Moreira et al., 2018), Adenocarcinoma cells of mammary gland (Huang et al., 2013; Sui et al., 2014, Zu et al., 2015), prostate cancer (Deng et al., 2015), HL-60 eukemic cells (Chen et al., 2002) and cells of cervical carcinoma (Srinivas et al., 2003). Though limited but there are some reports that suggest the role of emodin in restricting *in vivo* growth of Dalton's lymphoma via modulating enzymes of H_2O_2 metabolism (Singh and Trigun, 2013). Moreover, information is scanty on efficacy of emodin against melanoma.

III. PROPOSED APPROACH

In order to investigate cytotoxic potential of emodin against melanoma, this study compares IC_{50} value of emodin against MDA-MB-435S cells vs some other susceptible cell lines followed by delineating process of apoptosis in those melanoma cells.

IV. MATERIALS AND METHODS

A. In vitro studies on cell lines

Tumor cell lines, T47D and MDA-MB-435S were procured from NCCS, Pune, India and SiHa cells was gifted by Prof. G. Narayan, Molecular Human genetics, BHU, Varanasi. The cell lines were maintained in their respective medium containing 10% FBS and antibiotics; penicillin; 100 U/ml and streptomycin; 100 µg/ml in contamination free environment. The trypan blue exclusion test was applied to check cells viability.

B. MTT Assay (Kumari et al., 2011)

Briefly, 1 x 10^4 cells (SiHa, T47D and MDA-MB-435S) were seeded onto 96 well plates in 100 µl of the complete culture medium. The plates containing cells were then placed in a CO_2 incubator with 5% CO_2 at 37°C for 12 h. After overnight incubation was over, the cells were then treated with the emodin in a concentration range of 10 µM to 100 µM, prepared in the culture medium. The emodin treated cell plate was then incubated for another 24 h and 48 h. Cell viability was determined by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, that uses the principle of reducing a soluble yellow tetrazolium salt into blue formazan crystals by the live cells. After 24 h and 48 h schedule of emodin treatment, the MTT dye (10 µl/100 µl media made from 5 mg/ml stock) was re-constituted in phosphate buffered saline (PBS) and then added to all the wells. The plates were further allowed to be incubated for 3 - 4 h at 37°C in CO_2 incubator. The medium from each well was discarded post incubation and the formazan (purple-colour precipitate) formed was dissolved in 200 µl of dimethyl sulfoxide (DMSO). The plates were incubated for next 10 min with gentle shaking. Optical density was measured at 570 nm on synergy H1 hybrid microplate reader. The IC_{50} values of the emodin for each cell line were determined by constructing semi logarithmic dose–response plots, using Graphpad Prism5 software.

C. Ethidium Bromide / Acridine Orange staining (Kumar et al., 2016)

MDA-MB-435S cells (1 x 10^6cells/ml), seeded in 24 well plates (25,000 cells / well) for 24 h, were treated with 10, 20 and 40 µM emodin for next 24 h. Thereafter, cells were subjected to three washing with PBS followed by treatment with 5 mg/ml Acridine Orange and 3 mg/ml Ethidium Bromide stock solutions respectively for 2 min. The cells were again washed thrice with the PBS followed by re-suspending them in 50 µl PBS. The stained cell suspension (10 µl) was then placed on clean slides and images were captured immediately under fluorescence inverted microscope at 20X magnification with 480/30 nm excitation filter and 535/40 nm emission filter.

D. Comet assay (Fang et al., 2015)

The layering of gel for comet assay was done on pre-sterilized and clean microscope slides. Warm (100 µl) and normal melting (0.75%) agarose (NMPA) was smeared from one end of the slide to the other end with the help of another plain slide. The slides labeled for different samples were dried at 37°C. Simultaneously, in a micro centrifuge tube, 60 µl low melting agarose (LMPA) was added at 37°C to 20 µl of PBS containing MDA-MB-435S and mixed thoroughly by pipetting. After solidification of NMPA, the mixture of LMPA was dropped over the NMPA layer. To maintain uniform gel layering throughout, a cover slip was cautiously placed over the NMPA gel coat. The NMPA – LMPA gel was kept at 4℃ for solidification. After agarose layer was solidified, the cover slip was carefully removed and gently slides were immersed into a cold lysis solution (stock: 2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 0.3 M NaOH, 1% SDS, 1% Triton X – 100; pH 10). The lysis was done at 4°C, slides were removed from the lysis solution and gently placed in horizontal gel electrophoresis tank filled with the fresh cold electrophoresis buffer (300 mM NaOH / 1 mM EDTA; pH > 13). The slides were completely submerged in the buffer solution in such a way that there was no formation of air bubbles over the agarose gel. The slides dipped inside the alkaline buffer for a period of 30 min allowed the unwinding of DNA strands and exposed the alkali labile sites (alkali unwinding). The electrophoresis was carried out for 30 min at 4°C at 0.74 V/cm.

After electrophoresis, the slides were carefully shifted to a staining tray. The neutralizing Tris buffer (pH 7.4) was poured in the staining tray and slides were kept for 5 min. The buffer was then drained out and the process was repeated twice. The slides were then washed with distill water several times to remove the traces of neutralizing buffer. Each slide was then stained using 50 µl of Ethidium bromide (20 µg/ ml; 10X). The

excess stain from slides was blotted out using blotting sheet and a clean cover slip was placed over the gel. The EtBr stained slides were then visualized under fluorescent microscope, equipped with an excitation filter of A_{530}, emission filter of A_{590} at 40X magnification. The quantitative assessment of DNA damage in the cells was assessed by measuring the length of DNA migration and % of migrated DNA. This was quantified by measuring the tail – moment and olive – moment by open comet (v1.3.1) software.

E. Native PAGE for LDH assay

Non-denaturing PAGE was performed to analyze LDH isozymes following the protocol developed by Davis (1964) with some modifications as reported from our lab (Trigun et al., 2007). Briefly, in a mini gel cassette, 10% resolving gel was polymerized in 400 mM, Tris-Cl (pH 7.4) containing 4 µl TEMED and APS (0.1%). Stacking gel, prepared in 125 mM Tris-Cl (pH 6.8), was polymerized for loading the samples. Samples, were prepared in the loading dye constituted of 50 mM Tris-Cl (pH 6.8), 50% glycerol and bromophenol blue (0.0025%). The culture media (30 µl) was loaded in each lane and electrophoresis was carried out in non-SDS running buffer composed of Tris–Glycine buffer, pH 7.4. After electrophoresis the gels were placed in LDH specific activity staining mixture [(0.125 M Tris–Cl (pH 7.4), 0.5 mM $MgCl_2$, 0.1 mM Li-Lactate, 1 mg/ml NAD^+, 0.01 M NaCl, 0.25 mg/ml nitro blue tetrazolium (NBT), and 0.025 mg/ml phenazinemethosulfate (PMS)]. The LDH bands were allowed to appear gradually by gentle shaking and finally the gels were washed with distilled water; LDH bands were scanned at resolution of 300 dpi and finally quantitated by gel spectrometry using alpha imager 2200 gel documentation software. The isozymic bands in the gel were identified distinctively according to their pattern of migration shown by the tissue specific characteristic LDH bands (Mishra et al., 2004).

V. RESULTS

A. MTT assay

The cytotoxicity potentials of emodin were evaluated by MTT assay against different cell lines: SiHa; cervical cancer cell line, T47D; Breast cancer cell line and MDA-MB-435S; melanoma cancer cell line.

Table I: IC_{50} values of emodin against different cell lines:

Cell lines	24 h	48 h
SiHa	63.5 µM	59.0 µM
T47D	59.5 µM	58.6 µM
MDA-MB-435S	40.4 µM	32.4 µM

1) In vitro cytotoxicity of emodin against SiHa cell line

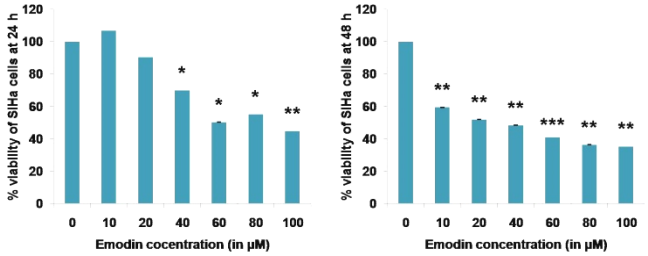


Fig.1 (a) Cytotoxicity of emodin against SiHa cells: Data represent cell viability after 24 h and 48 h treatment with different concentrations of emodin and presented as percent of surviving cells taking the value of the untreated control set as 100%. Values are given as mean ± SD (where, n= 3), *p<0.05, **p<0.01, *** p<0.001.

2) In vitro cytotoxicity of emodin against T47D cell line

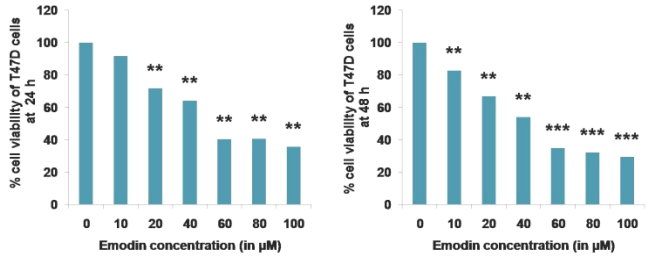


Fig.1 (b) Cytotoxicity of emodin against T47D cells: Data represent cell viability after 24 h and 48 h treatment with different concentrations of emodin and presented as percent of surviving cells taking the value of the untreated control set as 100%. Values are given as mean ± SD (where, n= 3), *p<0.05, **p<0.01, *** p<0.001.

3) In vitro cytotoxicity of emodin against MDA-MB-435S cell line

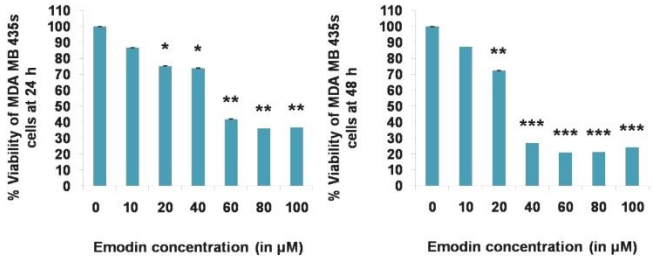


Fig.1 (c) Cytotoxicity of emodin against MDA-MB-435S cells: Data represent cell viability after 24 h and 48 h treatment with different concentrations of emodin and presented as percent of surviving cells taking the value of the untreated control set as 100%. Values are given as mean ± SD (where, n= 3), *p<0.05, **p<0.01, *** p<0.001.

The data from Figs 1.1, 1.2, 1.3 suggest that all the three cell lines tested showed a common pattern of linear decline up to 40 µM concentration of emodin followed by a static pattern thereafter up to 100 µM. However, a comparison of IC_{50} values given in table-1, though do not show much difference at 24 h and 48 h time points in case of SiHa & T47D cells, MDA-MB-435S cells showed a significant difference of IC_{50} values as a function of increasing incubation time. Also, IC_{50} values of

MDA-MB-435S cells at both the time points are markedly lower than the two other cell lines tested. Data clearly suggest greater cytotoxic potential of emodin against melanoma cell line than the SiHa & T47D cells.

B. Emodin dependent cell death pattern of MDA-MB-435S cells

1) Acridine Orange / Ethidium Bromide staining results

During this assay, acridine orange permeates into all cells making them to fluoresce green. On the other hand, Ethidium bromide interacts with the DNA of those cells which have lost their membrane integrity and thus, stains the nucleus red of the cells undergoing apoptosis.

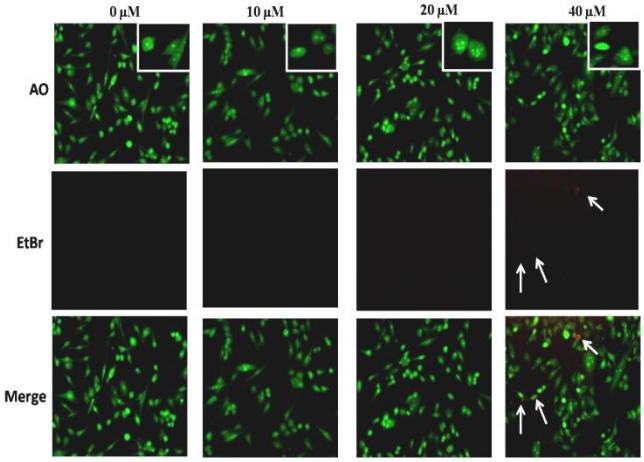
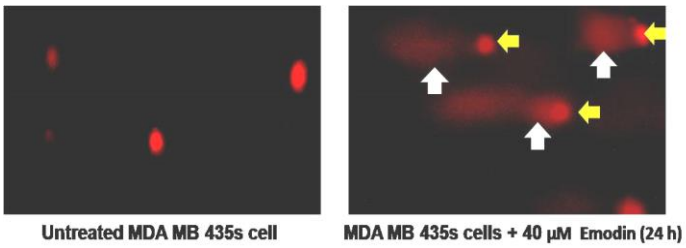


Fig. 2. EtBr/AO staining of MDA-MB-435S cells at increasing emodin concentrations (10, 20 and 40 µM) incubated for 24 h. The cells stained green, red and yellow correspond to live (acridine stained), dead (EtBr stained) and early apoptotic (yellow stained) cells respectively. The apoptotic and dead cells are marked with arrowheads. Photographs are the representative from the 3 repeat experiments.

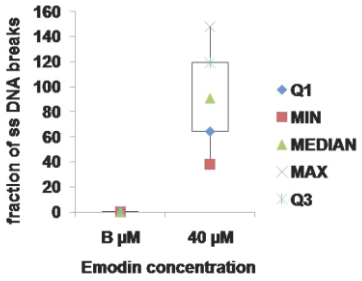
Accordingly, Acridine orange/ Ethidium Bromide fluorescence pattern of MDA-MB-435S cells treated 24 h with emodin (Fig. 2) showed the presence of apoptotic cells; early as well as late cell death stage and thus suggesting apoptotic potential of the emodin against this cell line. In the control (untreated) group sets, most of the cells are seen intact with normal nuclear membrane. However, at 10 and 20 µM emodin concentration, start of nuclear fragmentation could be seen (inset image for clarity). Moreover, at 40 µM of emodin, increased number of such cells with more intense nuclear fragmentation could be seen as a marker of advanced stage apoptotic cells.

2) Comet (Single-cell gel electrophoresis) Assay

Comet is the most relevant method for confirming apoptotic cell death mechanism, wherein, one can visualize the single /double – DNA strand breaks, alkali labile sites, DNA cross links and base– pair damages as confirmatory markers of apoptotic cell death. The treatment of agarose embedded cells initially with hypertonic lysis solution followed by the non ionic detergent causes breakdown of the cell membranes thereby releasing cytoplasm, nucleoplasm and finally dissolving the nucleosomes. The treatment of the leftover nucleoid with alkaline solutions relaxes/unwinds the DNA supercoils thereby exposing the alkali labile (apurinic / apyrimidinic) sites that appear as breaks. When subjected to electrophoresis, such breaks migrate faster towards the anode, thereby producing comet like appearance and length of the comet is considered indicative of apoptotic cell death.



(A)



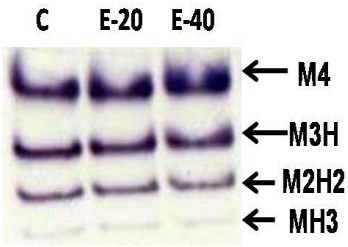
(B)

Fig.3. The figure (A) depicts typical comet with a bright head (indicated by yellow arrow) and tail (indicated by white arrow) in the cells treated up to 24 h with 40 µM emodin. The statistical analysis (figure B) shows significant increase of single strand break in emodin treated cells as compared to the untreated cells.

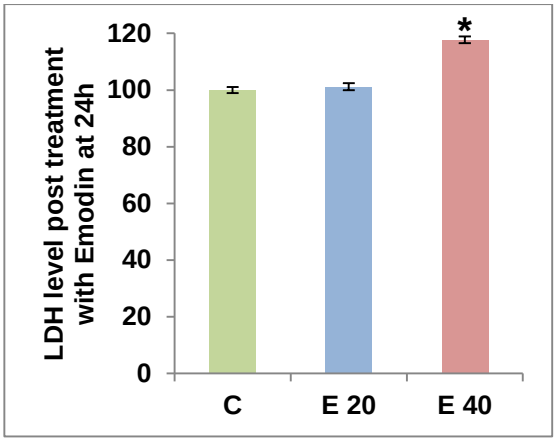
Comet assay result showed significant increase in single/double strand DNA break in cells as evident by the presence of comet tail in the 40 µM emodin treated cells.

3) Emodin depndent release of LDH from MDA-MB-435S cells at 24 h incubation

LDH is a cytosolic enzyme that exists in different isoforms and catalyzes the inter-conversion of pyruvate and lactate accompanied with the reversible oxidation of NADH vs NAD^+. It is released into the media from the cells when their permeability is lost. The level of serum (LDH), thus, can be used as a sensitive prognostic parameter in determining the cellular metabolic state, representing activation and malignant transformation of the cell undergoing cancer progression.



(A)



(B)

Fig.4. The emodin dependent release of LDH in culture medium of MDA-MB-435s cells after 24 h. (A) Represents LDH activity stained gel photograph from three 10% PAGE repeats with 30 µl of medium loaded in each lane. B represents relative intensityof the LDH bands based on densitometric analysis of 3 PAGE repeats (*p<0.05).

Out of the 3 detectable LDH isozymes, intensity of M4 type LDH, known as a marker of tumor growth, shows emodin concentration dependent gradual increase in the MDA-MB-435S cells culture medium of (Fig.4).

VI. DISCUSSION

The potential of emodin could previously been explored as traditional Chinese medicine in the treatment of gallstones, hepatitis, osteomyelitis, skin burns, infection and inflammation. As inflammation constitutes a common event during *in vivo* tumor progression, it is argued to examine this compound for its anti carcinogenic properties as well. Indeed, in an *in vivo* model of DL in mice, emodin has been found to regress the tumor by modulating ROS metabolizing enzymes (Singh & Trigun, 2013). The present study was conducted to evaluate its cytotoxic pattern in different cell lines followed by investigation of apoptotic cell death in case of a melanoma (MDA-MB-435S) cells. MTT is a colorimetric assay that measures cell viability in terms of recording mitochondrial dehydrogenase activity. This assay was employed to first calculate IC_{50} values of the emodin against three different mammalian cancer cells (Cervical, Breast and Melanoma cells) with an aim to assess its broad range anti cell proliferative activity *in vitro*. The results show a significant decline, in the number of viable SiHa, T47D and MDA-MB-435S cells when treated with different concentrations of emodin. Out of these three cell lines tested, emodin exhibited only a moderate level of cytotoxicity against SiHa and T47D cells with no further effect of the increasing incubation time. However, a stronger cytotoxic potential could be seen against the melanoma MDA-MB-435S cells exhibiting the lowest IC_{50} value in the range of 30 – 40 µM following an increasing pattern as a function of incubation time. Moreover, the IC_{50} value of emodin against the cell lines reported herein, corroborated the IC_{50} range recorded in case of some other mammalian cancer cells (He et al., 2012; Li et al., 2014; Li et al., 2016; Zheng et al. 2019). Thus, it is argued that emodin possess a wide range of cytotoxic potential against several cell lines and thereby advocating about exploring the mechanism by which it causes cell death in case of the most affected melonoma cell line.

A dual staining method of Acridine Orange (AO) and Ethidium Bromide (EtBr) is considered as a reliable tool to ascertain the mechanism of cell death *in vitro*. Acridine orange stains both, the cytoplasm and the nucleus and emits green fluorescence. Whereas, Ethidium Bromide easily permeates in the cells undergoing apoptosis, wherein, it intercalates with the minor groove of DNA inside the nucleus (Widlak et al., 2002), and emits red fluorescence. As a result, the nucleus of the cells, which is in the process of early and late apoptosis, shows yellow coloration as a result of co-localization of AO/EtBr fluorescence. In the present study, MDA-MB-435S cells treated with the increasing concentrations of emodin (10, 20 and 40 µM) when subjected to AO/EtBr staining showed no change in morphology of nucleus in the control and 10 µM set, thus indicating no cell death in the untreated MDA-MB-435S cells. However, at 20 µM and 40 µM emodin, a concentration dependent increase in nuclear fragmentation (showing yellow coloured nuclei could be observed; Fig. 2) and thereby, suggesting a number of cells undergoing apoptosis due to emodin treatment. Moreover, some cells showing red coloured nuclei at higher concentration suggest about late stage apoptosis/ start of necrosis as well. Taking together, it is evident that strong cytotoxic potential of emodin is accompanied with the initiation of apoptosis in MDA-MB-435S cells *in vitro*.

The argument gets support from findings of Fig.3; showing the DNA damage through comet assay. Herein, as compared to the untreated MDA-MB-435S cells, the emodin-treated cells showed appearance of more number of low molecular weight DNA fragments observed in the form of the comet tail. Some reports do suggest about similar effects of emodin against human hepatoma HepG2 and C3A cells (Shieh et al., 2004), the leukemic HL-60 cells (Chen et al., 2002), the Bu 25TK cervical cancer cells (Srinivas et al., 2003) and MDA-MB-231 & HSC5 cells (Huang et al., 2004). Thus the findings of Fig 3 provide confirmatory evidence about pro-apoptotic potential of emodin against MDA-MB-435S cells *in vitro*.

LDH is a tetrameric enzyme, however, its five different isozymes show tissue specificity in most of the mammalian tissues and therefore, it is now considered as one of the parameters to study tumor progression. In growing tumors, pyruvate is catalyzed to generate lactate by LDH because of impaired mitochondrial function and hyperglycolytic syndrome acquired by the tumor cells (Koiri et al, 2011). The LDH isozymes have been reported to show differential expressions pattern in different types of tumors (Uchide et al., 2009; Maes et at., 2015; Hajiaghaalipour et al., 2017; Kumar et al., 2018). Wherein, over expression of M4 type LDH has been reported to associate with tumor progression (Koiri et al, 2009, 11, 13). Similarly, increased amount of M4 LDH release in culture media of the tumor cells could be interpreted as a biochemical marker for cell death (Koiri et al, 2009). Thus, the findings of Fig 4, wherein selectively M4 LDH has been shown to be increased in the media due to emodin treatment, could be inferred as emodin dependent death of MDA-MB-435S cell *in vitro*. Taking together, the findings presented advocate that emodin induces apoptosis to produce strong cytotoxicity against MDA-MB-435S cells.

CONCLUSIONS

Based on the results of *in vitro* experiments it is evident that emodin, considered to be a non genotoxic and non mutagenic natural compound, is able to cause toxicity to a number of cell lines, however, with a lower IC_{50} values for MDA-MB-435S cells. This is consistent with the findings supporting apoptotic induction in these MDA-MB-435S cells. The findings suggest emodin as a promising compound for its evaluation against the animal models with squamous / basal cell carcinoma.

FUTURE DIRECTIONS

Emodin could be investigated for its therapeutic applications against skin cancer in the animal models.

ACKNOWLEDGEMENT

The work was financially supported by ICMR- JRF to SY. The instrumentation facilities out of ICMR project (P 07/253) to SKT and from DIST FIST program in Zoology & DBT-ISLS unit in the Institute of Science, BHU, are also acknowledged. Sincere thanks to Dr. Krishna Beer Singh, Department of Pharmacology and Chemical Biology, School of Medicine, University of Pittsburgh, for initial training on cell culture techniques. A generous gift of SiHa cells from Prof. G. Narayan, Molecular Human genetics, BHU, is also acknowledged.

REFERENCES

Ali, Z., Yousaf, N., Larkin, J. (2013) Melanoma epidemiology, biology and prognosis. European Journal of Cancer Supplement, 11(2): 81–91.

Chen, G., Zhang, J., Zhang, H., Xiao, Y., Kao, X., Liu, Y., Liu, Z. (2015) Anti-inflammatory effect of emodin on lipopolysaccharide-induced keratitis in Wistar rats. International Journal of Clinical and Experimental Medicine, 8(8):12382-9.

Chen, Y. C., Shen, S. C., Lee, W. R., Hsu, F. L., Lin, H. Y., Ko, C. H., Tseng, S. W. (2002) Emodin induces apoptosis in human promyeloleukemic HL-60 cells accompanied by activation of caspase 3 cascade but independent of reactive oxygen species production. Biochemical Pharmacology, 64(12):1713-24.

Chen, Y. Y., Chiang, S. Y., Lin, J. G., Ma, Y. S., Liao, C. L., Weng, S. W., Lai, T. Y., Chung, J. G. (2010) Emodin, aloe-emodin and rhein inhibit migration and invasion in human tongue cancer SCC-4 cells through the inhibition of gene expression of matrix metalloproteinase-9. International Journal of Oncology, 36(5):1113-20.

Chien, S. C., Wu, Y. C., Chen, Z. W., Yan, W. C. (2015) Naturally Occurring Anthraquinones: Chemistry and Therapeutic Potential in Autoimmune Diabetes. Evidence Based Complementary Alternative Medicine, 2015: 357357.

Chihara, T., Shimpo, K., Beppu, H., Yamamoto, N., Kaneko, T., Wakamatsu, K., Sonoda, S. (2015) Effects of Aloe-emodin and Emodin on Proliferation of the MKN45 Human Gastric Cancer Cell Line. Asian Pacific Journal of Cancer Prevention, 16(9):3887-91.

Cui, Y., Lu, P., Song, G., Liu, Q., Zhu, D., Liu, X. (2016) Involvement of PI3K/Akt, ERK and p38 signaling pathways in emodin-mediated extrinsic and intrinsic human hepatoblastoma cell apoptosis. Food and Chemical Toxicology, 92:26-37.

Deitersen, J., El-Kashef, D. H., Proksch, P., Stork, B. (2019) Anthraquinones and autophagy - Three rings to rule them all? Bioorganic and Medicinal Chemistry, 8:115042.

Deng, G., Ju, X., Meng, Q., Yu, Z. J., Ma, L. B. (2015) Emodin inhibits the proliferation of PC3 prostate cancer cells in vitro via the Notch signaling pathway. Molecular Medicine Report, 12(3):4427-4433.

Dong, X., Fu, J., Yin, X., Cao, S., Li, X., Lin, L., Huyiligeqi, N. J. (2016) Emodin: A Review of its Pharmacology, Toxicity and Pharmacokinetics. Phytotherapy Research, 30(8):1207-18.

Dong, X., Ni, B., Fu, J., Yin, X., You, L., Leng, X., Liang, X., Ni, J. (2018) Emodin induces apoptosis in human hepatocellular carcinoma HepaRG cells via the mitochondrial caspase-dependent pathway. Oncology Reports, 40(4):1985-1993.

Fang, L., Neutzner, A., Turtschi, S., Flammer, J., Mozaffarieh, M. (2015) Comet assay as an indirect measure of systemic oxidative stress. Journal of Visualized Experiments, (99):e52763.

Hajiaghaalipour, F., Bagheri, E., Faraj, F. L., Abdulla, M. A., Majid, N. A. (2017) Underlying mechanism for the modulation of apoptosis induced by a new benzoindole derivative on HT-29 colon cancer cells. Royal Society of Chemistry Advances, 7, 38257.

Hanahan, D., Weinberg, R. A. (2000) The hallmarks of cancer. Cell, 100(1):57-70.

He, L., Bi, J. J., Guo, Q., Yu, Y., Ye, X. F. (2012) Effects of emodin extracted from Chinese herbs on proliferation of non-small cell lung cancer and underlying mechanisms. Asian Pacific Journal of Cancer Prevention, 13(4):1505-10.

Homsi, J., Kashani-Sabet, M., Messina, J. L., Daud, A. (2005) Cutaneous melanoma: prognostic factors. Cancer Control, 12(4):223-9.

Howlader, N., Noone, A. M., Krapcho, M., Miller, D., Brest, A., Yu, M., Ruhl, J., Tatalovich, Z., Mariotto, A., Lewis, D.R., Chen, H.S., Feuer, E. J., Cronin, K. A. (2019) SEER Cancer Statistics Review, 1975-2016, National Cancer Institute, https://seer.cancer.gov/csr/1975_2016/

Huang, P. H., Huang, C. Y., Chen, M. C., Lee, Y. T., Yue, C. H., Wang, H. Y., Ho, L. (2013) Emodin and Aloe-Emodin Suppress Breast Cancer Cell Proliferation through ERα Inhibition. Evidence Based Complementary and Alternative Medicine, 2013:376123.

Huang, Q., Shen, H. M., Ong, C. N. (2004) Inhibitory effect of emodin on tumor invasion through suppression of activator protein-1 and nuclear factor-kappaB. Biochemcal Pharmacology, 68(2):361-71.

Koiri, R. K., Trigun, S. K. (2011) Dimethyl Sulfoxide Activates Tumor Necrosis factorα-p53 Mediated Apoptosis and Down Regulates D-fructose-6-phosphate-2-kinase and Lactate dehydrogenase-5 in Dalton's Lymphoma in Vivo. Leukemia Research, 35(7):950-6.

Koiri, R. K., Mehrotra, A., Trigun, S. K. (2013) Targetting Cancer With Ru(III/II)-phosphodiesterase Inhibitor Adducts: A Novel Approach in the Treatment of Cancer. Medical Hypotheses, 80(6):841-6.

Koiri, R. K., Trigun, S. K., Mishra, L., Pandey, K., Dixit, D., Dubey, S. K. (2009) Regression of Dalton's Lymphoma in vivo via Decline in Lactate Dehydrogenase and Induction of Apoptosis by a ruthenium(II)-complex Containing 4-carboxy N-ethylbenzamide as Ligand. Investigational New Drugs, 27(6):503-16.

Kumar, A., Kumar, A., Gupta, R. K., Paitandi, R. P., Singh, K. B., Trigun, S. K., Hundal, M. S., Pandey, D. S. (2016) Cationic Ru(II), Rh(III) and Ir(III) complexes containing cyclic π-perimeter and 2-aminophenyl benzimidazole ligands: Synthesis, molecular structure, DNA and protein binding, cytotoxicity and anticancer activity. Journal of Organometallic Chemistry, 801, 68-79

Kumar, P., Nagarajan, A., Uchil, P. D. (2018) Analysis of Cell Viability by the Lactate Dehydrogenase Assay. Cold Spring Harbor Protocol, 2018(6). doi: 10.1101/pdb.prot095497.

Kumari, N., Maurya, B. K., Koiri, R. K., Trigun, S. K., Saripella, S., Coogan, M. P., Mishra, L. (2011) Cytotoxic activity, cell imaging and photocleavage of DNA induced by a Pt (II) cyclophane bearing 1, 2 diamino ethane as a terminal ligand. Medicinal Chemistry Communication, **2**, 1208-1216.

Li, W. Y., Ng, Y. F., Zhang, H., Guo, Z. D., Guo, D. J., Kwan, Y. W., Leung, G. P., Lee, S. M., Yu, P. H., Chan, S. W. (2014) Emodin elicits cytotoxicity in human lung adenocarcinoma A549 cells through inducing apoptosis. Inflammopharmacology, 22(2):127-34.

Li, X., Wang, H., Wang, J., Chen, Y., Yin, X., Shi, G., Li, H., Hu, Z., Liang, X. (2016) Emodin enhances cisplatin-induced cytotoxicity in human bladder cancer cells through ROS elevation and MRP1 downregulation. BioMed Central Cancer, 16:578.

Lin, W., Zhong, M., Yin, H., Chen, Y., Cao, Q., Wang, C., Ling, C. (2016) Emodin induces hepatocellular carcinoma cell apoptosis through MAPK and PI3K/AKT signaling pathways in vitro and in vivo. Oncology Reports, 36(2):961-7.

Liu, B., Yuan, B., Zhang, L., Mu, W., Wang, C. (2015) ROS/p38/p53/Puma signaling pathway is involved in emodin-induced apoptosis of human colorectal cancer cells. International Journal of Clinical and Experimental Medicine, 8(9):15413-22.

Liu, Y., Wang, Q., Yang, J., Guo, X., Liu, W., Ma, S., Li, S. (2018) Polygonum multiflorum Thunb.: A Review on Chemical Analysis, Processing Mechanism, Quality Evaluation, and Hepatotoxicity. Frontiers in Pharmacology, 2018; 9: 364.

Ma, L., Yang, Y., Yin, Z., Liu, M., Wang, L., Chen, L., Zhu, L., Yan, G. H. (2017) Emodin suppresses the nasopharyngeal carcinoma cells by targeting the chloride channels. Biomedicine and Pharmacotherapy, 90:615-625.

Ma, Q., Ding, Y., Wu, Z., Li, Y. (2018) Antitumor effects of emodin in CACO-2 human colon carcinoma cells are mediated via apoptosis, cell cycle arrest and downregulation of PI3K/AKT signaling Pathway. Journal of the Balkan Union of Oncology, 23(3):587-591.

Maes, M., Vanhaecke, T., Cogliati, B., Yanguas, S. C., Willebrords, J., Rogiers, V., Vinken, M. (2015) Measurement of Apoptotic and Necrotic Cell Death in Primary Hepatocyte Cultures. Methods in Molecular Biology, 1250:349-61.

Mishra, L., Singh, A. K., Trigun, S. K., Singh, S. K., Pandey, S. M. (2004) Anti-HIV and cytotoxic ruthenium(II) complexes containing flavones: biochemical evaluation in mice. Indian Journal of Experimental Biology, 42(7):660-6.

Monisha, B. A., Kumar, N., Tiku, A. B. (2016) Emodin and its role in chronic diseases. Advances in Experimental Medicine and Biology, 928:47–73.

Moreira, T. F., Sorbo, J. M., Souza, F. O., Fernandes, B. C., Marins, Ocampos F. M., Oliveira, D. M. S., Arcaro, C. A., Assis, R. P., Barison, A., Miguel, O. G., Baviera, A. M., Soares, C. P., Brunetti, I. L. (2018) Emodin, Physcion, and Crude Extract of *Rhamnus sphaerosperma* var. *pubescens* Induce Mixed Cell Death, Increase in Oxidative Stress, DNADamage,and Inhibition of AKT in Cervical and Oral Squamous Carcinoma Cell Lines. Oxidative Medicine and Cellular Longevity, 2018: 2390234.

National Toxicology Program (2001) NTP Toxicology and Carcinogenesis Studies of EMODIN (CAS NO. 518-82-1) Feed Studies in F344/N Rats and B6C3F1 Mice. National Toxicology Program Technical Report Ser, 493:1-278.

Rastrelli, M., Tropea, S., Rossi, C. R., Alaibac, M. (2014) Melanoma: epidemiology, risk factors, pathogenesis, diagnosis and classification, In Vivo. 28(6):1005-11.

Shieh, D. E., Chen, Y. Y., Yen, M. H., Chiang, L. C., Lin, C. C. (2004) Emodin-induced apoptosis through p53-dependent pathway in human hepatoma cells. Life Sciences, 74(18):2279-90.

Singh, K. B., Trigun, S. K. (2013) Apoptosis of Dalton's lymphoma due to in vivo treatment with emodin is associated with modulations of hydrogen peroxide metabolizing antioxidant enzymes. Cell Biochemistry and Biophysics, 67(2):439-49.

Srinivas, G., Anto, R. J., Srinivas, P., Vidhyalakshmi, S., Senan, V. P., Karunagaran, D. (2003) Emodin induces apoptosis of human cervical cancer cells through poly(ADP-ribose) polymerase cleavage and activation of caspase-9. European Journal of Pharmacology, 473(2-3):117-25.

Srinivas, G., Babykutty, S., Sathiadevan, P. P., Srinivas, P. (2007) Molecular mechanism of emodin action: transition from laxative ingredient to an antitumor agent. Medicinal Research Reviews, 27(5):591-608.

Su, J., Yan, Y., Qu, J., Xue, X., Liu, Z., Cai, H. (2017) Emodin induces apoptosis of lung cancer cells through ER stress and the TRIB3/NF-κB pathway. Oncology Reports, 37(3):1565-1572.

Su, Y. T., Chang, H.L., Shyue, S. K., Hsu, S. L. (2005) Emodin induces apoptosis in human lung adenocarcinoma cells through a reactive oxygen species-dependent mitochondrial signaling pathway. Biochemcal Pharmacology, 70(2):229-41.

Sui, J. Q., Xie, K. P., Zou, W., Xie, M. J. (2014) Emodin inhibits breast cancer cell proliferation through the ERα-MAPK/Akt-cyclin D1/Bcl-2 signaling pathway. Asian Pacific Journal of Cancer Prevention, 15(15):6247-51.

Tang, Q., Wu, J., Zheng, F., Hann, S. S., Chen, Y. (2017) Emodin Increases Expression of Insulin-Like Growth Factor Binding Protein 1 through Activation of MEK/ERK/AMPKα and Interaction of PPARγ and Sp1 in Lung Cancer. Cell Physiology and Biochemistry, 41(1):339-357.

Trigun, S. K., Koiri, R. K., Mishra, L., Dubey, S. K., Singh, S., Pandey, P. (2007) Ruthenium Complex as Enzyme Modulator: Modulation of Lactate Dehydrogenase by a Novel Ruthenium(II) Complex Containing 4-Carboxy N-Ethylbenzamide as a Ligand. Current Enzyme Inhibition, 3, 243-253(11).

Uchide, N., Ohyama, K., Bessho, T., Toyoda, H. (2009) Lactate dehydrogenase leakage as a marker for apoptotic cell degradation induced by influenza virus infection in human fetal membrane cells. Intervirology, 52(3):164-73.

Wang, Y., Luo, Q., He, X., Wei, H., Wang, T., Shao, J., Jiang, X. (2018) Emodin Induces Apoptosis of Colon Cancer Cells via Induction of Autophagy in aROS-Dependent Manner. Oncology Research, 26(6):889-899.

Wang, Z., Yang, L., Fan, H., Wu, P., Zhang, F., Zhang, C., Liu, W. and Li, M. (2017) Screening of a natural compound library identifies emodin, a natural compound from Rheum palmatum Linn that inhibits DPP4. PeerJ. 2017; 5: e3283.

Widlak, P., Palyvoda, O., Kumala, S., Garrard, WT. (2002) Modeling Apoptotic Chromatin Condensation in Normal Cell Nuclei. Requirement for Intranuclear Mobility and Actin Involvement. Journal of Biological Chemistry, 277(24):21683-90.

Xie, M. J., Ma, Y. H., Miao, L., Wang, Y., Wang, H. Z., Xing, Y. Y., Xi, T., Lu, Y. Y. (2014) Emodin-provoked oxidative stress induces apoptosis in human colon cancer HCT116 cells through a p53-mitochondrial apoptotic pathway. Asian Pacific Journal of Cancer Prevention, 15(13):5201-5.

Yang, Y. C., Lim, M. Y. and Lee, H. S. (2003) Emodin Isolated from Cassia obtusifolia (Leguminosae) Seed Shows Larvicidal Activity against Three Mosquito Species. Journal of Agricultural and Food Chemistry, 51, 7629−7631.

Ying, J., Xu, H., Wu, D., Wu, X. (2015) Emodin induces apoptosis of human osteosarcoma cells via mitochondria- and endoplasmic reticulum stress-related pathways. International Journal of Clinical and Experimental Pathology, 8(10):12837-44.

Zhang, K., Jiao, K., Zhu, Y., Wu, F., Li, J., Yu, Z. (2015) Effect of emodin on proliferation and cell cycle of human oral Squamous carcinoma Tca8113 cells in vitro. Nan Fang Yi Ke Da Xue Xue Bao, 35(5):665-70.

Zhang, L., He, D., Li, K., Liu, H., Wang, B., Zheng, L., Li, J. (2017) Emodin targets mitochondrial cyclophilin D to induce apoptosis in HepG2 cells. Biomedicine & Pharmacotherapy, 90:222-228.

Zhao, L., Fei, W., Liang, J. C., Hai,- R. X. , Yuan,- Y. L., Fan, L., Wei, H., Hong, X., Zhan, -Q. Y. (2013) In Vitro and in Vivo Studies of the Inhibitory Effects of Emodin Isolated from Polygonum cuspidatum on Coxsakievirus B4. Molecules, 18, 11842-11858.

Zheng, X. Y., Yang, S. M., Zhang, R., Wang, S. M., Li, G. B., Zhou, S. W. (2019) Emodin-induced autophagy against cell apoptosis through the PI3K/AKT/mTOR pathway in human hepatocytes. Drug Design Development and Therapy, 13:3171-3180.

Zhou, R., Wang, X. W., Sun, Q., Ye, Z. J., Liu, J., Zhou, D. H., Tang, Y. (2019) Anticancer Effects of Emodin on HepG2 Cell: Evidence from Bioinformatic Analysis. Biomed Research International, 2019: 3065818.

Zu, C., Zhang, M., Xue, H., Cai, X., Zhao, L., He, A., Qin, G., Yang, C., Zheng, X. (2015) Emodin induces apoptosis of human breast cancer cells by modulating the expression of apoptosis-related genes. Oncology Letters, 10(5):2919-2924.

***