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RESEARCH ARTICLE

PRODIGIOSIN INDUCED APOPTOSIS AND INHIBITED PROLIFERATION IN CARCINOMA HSC-2 CELLS

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ABSTRACT

The present study aims to investigate the anti-proliferative, apoptotic properties of prodigiosin, using a human oral squamous cell carcinoma HSC-2 cell line as a model system. HSC- 2 cells were cultured in the presence of prodigiosin at various concentrations (1–30µg/ml) for 48 h and the percentage of cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The results showed that prodigiosin inhibited the cells viability in time and concentration dependent characteristics at different concentrations. We found that anti-proliferative effect of prodigiosin was associated with apoptosis on HSC-2 cells by determinations of DNA fragmentation, Hoechst 33258 staining, caspases activity, and TNF-α was significantly changed when compare DMSO control group. In addition, activity of lactate dehydrogenase (LDH) release increased when the cells incubated with prodigiosin at various concentrations and times. These results suggested that prodigiosin treatment inhibited proliferation and induction of apoptosis in human oral squamous cell carcinoma HSC-2.

INTRODUCTION

Cancer is the second leading cause of death in the United States, and cancer in oral cavity is one of the fifth most common cancer worldwide, it is a major cause of morbidity and mortality. Despite advances in cancer detection and treatment, the 5-year survival rate is the lowest among of all major cancers. (Argiris *et al*., 2008, Felthaus *et al*., 2011). The high incidence of oral cancer in the Indian subcontinent is causally associated with the widespread habit of tobacco and betel quid chewing. Betel quid chewing induces physical abrasions, which might create a mitogenic environment. In addition, chronic exposure of the oral cavity to the mutagenic constituents of betel quid may cause mutations in cellular genes (Kaur *et al*., 2000, Ashburner and Bonner, 1979). The risk factors associated with oral cancer may exert their effects via regulation of cell proliferation and anti apoptotic pathways. However, therapeutic strategies to manipulate apoptosis have therefore gained immense potential and several viable drug targets identified in recent years (Ma *et al*., 2009). Agents that are capable of inducing selective apoptosis of cancer cells are being given great interest in developing novel cancer preventive approaches (Cragg *et al*., 2009).

Natural antitumor drugs derived from organisms have been proven effective and less toxic for cancer therapy (Ma *et al*., 2009, Ravelo *et al.,* 2004). Promising natural products should be more than synthetic drugs by the huge distribution of the earth. Large-scale screenings of microorganisms, plants, animals and marine organisms for anti-tumor drugs have been performed in recent decades (Cragg *et al*., 2009). .However, production of secondary metabolites from microorganisms with potential anti-tumor activities have been discovered in recent years.

Prodigiosins (PGs) (2-methyl- 3-pentyl- 6-methoxy prodigiosene) is a red pigment secondary metabolite produced by *Serratia* sp. and other unrelated microbial strains such as *Vibrio psychroerythrus, streptomyces griseoviridis* and *Hahella chejuensis*. PGs have a common pyrrolyldipyrrolylmethene (PPM) skeletal core with different alkyl substituents such as linear carbon chain and cyclic derivatives that form PG derivatives, i.e., a family of naturally secreting pyrrole red pigments that are secondary metabolites from microorganisms (Fürstner, 2003, Pandey *et al.,* 2009). Prodigiosin is known to anti microbial, antimalarial, immunosuppressive, antifungal, proapoptotic antiproliferative actions. Hence the aim our present study was to investigate the anti proliferative effect of prodigiosin (PG), a secondary metabolite product against human oral cancer cells HSC-2.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM), 0.25% trypsin-EDTA solution, sodium bicarbonate solution, bovine serum albumin (BSA), 3-[4,5-dimethythiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT), propidium iodide, ethidium bromide, acridine orange, rhodamine 123, agarose,

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Fetal bovine serum (FBS) and antibiotic/antimycotic solution were from Gibco (Gibco, USA). Sodium phosphate (monobasic and dibasic), sodium chloride, sodium hydroxide, sodium carbonate, hydrochloric acid and methanol were purchased from Sisco Research Laboratories (Mumbai, India).

Prodigiosin (PGs) purification

2-Methyl-3-pentyl-6-methoxyprodigiosene (PG) was purified from *Serratia marcescens*, as previously Described (Montaner *et al.,* 2000). It was then solubilized and its concentration determined by UV–vis in 95% EtOH–HCl (2535 = 112000/ M cm).

Cell culture

The HSC-2 cell line was procured from the National Centre for Cell Science (Pune, India). Cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS. Upon reaching confluence, cells were detached using Trypsin-EDTA solution.

Cell proliferation assay

HSC-2 Cells were cultured in DMEM medium supplemented with 5% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in an incubator at 37ºC and 5% CO_2. The cells with medium changed every other day were subcultured at every 3 days, and were then plated at an appropriate density according to each experimental scale. Cells were used for experiments within eight passages to ensure cell line stability. Stock of cells were routinely frozen and stored in liquid N_2. To study the effect of prodigiosin on HSC-2 cells were seeded in 24-well culturing plates at a density of 5×10^4 cells/well in 1mL of culture medium. The cells were divided into three groups as followings: (1) DMSO control group; (2) Prodigiosin group supplemented at final prodigiosin concentrations of 1, 5, 10, 20, and 30 µg/ml respectively. Each sample had at least three replicates. Cells were cultured for 12, 24, or 48 h separately before thiazol blue was added to assay the viability by MTT method. Absorbance at 570 nm was read and cell viability (%) was calculated as (A570 of drug-treated sample/A570 of control) ×100 (shen *et al.,* 1995). Based on MTT assay, we selected doses 1, 5 and 10 µg/ml prodigiosin treatment for 12, 24 and 48h in further studies.

Lactatedehydrogenase (LDH) activity

HSC-2 injury was quantitated by measuring the lactate dehydrogenase (LDH) released from lysed cells into the bathing medium, utilizing a commercial available kit (Papadopoulos *et al.,* 1997).Total LDH release corresponding to complete HSC- 2 cells death was determined at the end of each experiment following freezing at −70ºC and rapid thawing. LDH release (%) = (LDH activity in media)/ (LDH activity in media +LDH activity in total death cells) ×100%.

DNA agarose gel electrophoresis

DNA extraction and agarose gel electrophoresis were performed using the following method. Briefly, $1X10^6$ cells were plated in 100-mm Petri dishes with DMEM containing 10% FBS. Cells were incubated for 24 h in 5% CO2 and 95% air at 37ºC. Control cells received 0.1% DMSO containing DMEM, and prodigiosin-treated cells received 1, 5 and 10µg/ml of prodigiosin-containing DMEM. After 24 h, the cells were trypsinized and combined with the cells in the medium by centrifugation at 1500 r.p.m. for 5 min, then they were washed twice with PBS. The resulting pellet was resuspended in 0.25 ml of lysis buffer, transferred to a microfuge tube, and incubated for 1 h at 37 ^{o}C. To this 4µl of proteinase K was added and tubes were then incubated at 50^{o} C for 3 h. To each tube, 0.5 ml of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added, mixed and centrifuged at 13 000 r.p.m. for 30 min at 4° C to separate the DNA containing upper aqueous phase. To the resultant aqueous phase, two volumes of ice-cold absolute ethanol and 1/10 the volume of 3m sodium acetate were added and kept at –20°C overnight to precipitate DNA. The DNA was pelleted by centrifuging at 13 000 r.p.m. for 10 min at 4°C and the supernatant was aspirated and the pellet washed in 1 ml of 70% ethanol. After repeating the above centrifugation step and removing last traces of the supernatant fraction, the pellet was allowed to dry at room temperature for approximately 30 min before being resuspended in 50 µl of Tris-EDTA buffer. DNA was quantified by ultraviolet visible spectroscopy and 10µg of DNA was electrophoresed in 1.5% agarose gel containing ethidium bromide in a mini gel tank containing Tris-borate-EDTA buffer for 2 h at 90 V. The gel was then examined under ultraviolet light and photographed.

Fluorescent staining of nuclei with Hoechst 33258 staining

The nuclei of HSC2 cells were stained with chromatin dye (Hoechst 33258) (Du *et al.,* 2007). The cells were fixed with 3.7% paraformadehyde for 10 min, washed twice with PBS, and incubated with 10 µM Hoechst 33258 in PBS at room temperature for 30 min. After three washes, the cells were observed under a fluorescence microscope.

Activity of caspase 3 and 9 in chromogenic method

Activities of caspase-3 and caspase-9 were assessed by CasPASETM Apoptosis Assay Kit as manufacturer instructions (Catalog Nos. 786-202A and 786-205A were purchased from Geno Technology, St. Louis, MO, USA).

Assay of TNF- α secretion

TNF- α concentration in the cell-derived culture supernatants was measured by a modified ELISA, as described by (Kim and Lee, 1999). The ELISA was devised by coating 96- well plates of human monoclonal antibody with specificity for human TNF- α. Before use and between subsequent steps in the assay, coated plates were washed with PBS containing 0.05% Tween-20. All reagents used in this assay were incubated for 2 h at 37ºC. Recombinant human TNF-α were diluted and used as a standard. Serial dilutions starting from 1 pg/ml were used to establish the standard curve. Assay plates were exposed sequentially to rabbit anti-TNF- α antibody, and phosphatase conjugated goat anti-rabbit IgG antibody and avidine peroxidase, and *p*-nitrophenyl phosphate and ABTS substrate solution containing 30% H_2O_2. The plates were read at 405 nm.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) by using statistical package of social science (SPSS) version 10.0 for windows. The values are mean ± SD for six samples in each group. P values≤0.05 were considered as significant.

RESULT

Inhibition of cell growth by prodigiosin

Effects of Prodigiosin on the growth of HSC-2 cells were investigated by MTT method. HSC-2 cells were not affected when exposed to low concentrations of prodigiosin (1 µg), and the cells exposed to high concentrations of prodigiosin (5-10 µg) revealed that cell proliferation was inhibited. [that is, increase in dose and time dependent resulted in increased inhibition $p<0.05$ of proliferation]. Based on this study, we selected 1, 5 and 10 µg/ml prodigiosin for further procedure at different time period up to 48h Fig. 1.

Influence of prodigiosin in TNF-α degeneration

Prodigiosin activate the release of TNF-α by 70–90% and there was also a significant difference in both the dose and time treatments of prodigiosin. At 10 ug/ml concentration significantly activate TNF-α expression. It was found at 24 and 48 h of incubation period. The 1 ug/ml concentration does not change the level after 48 h incubation. When compared untreated control and 5 ug/ml concentration showed 2-30 % of activation at 24 and 48 h incubated treatment regimen Fig. 3.

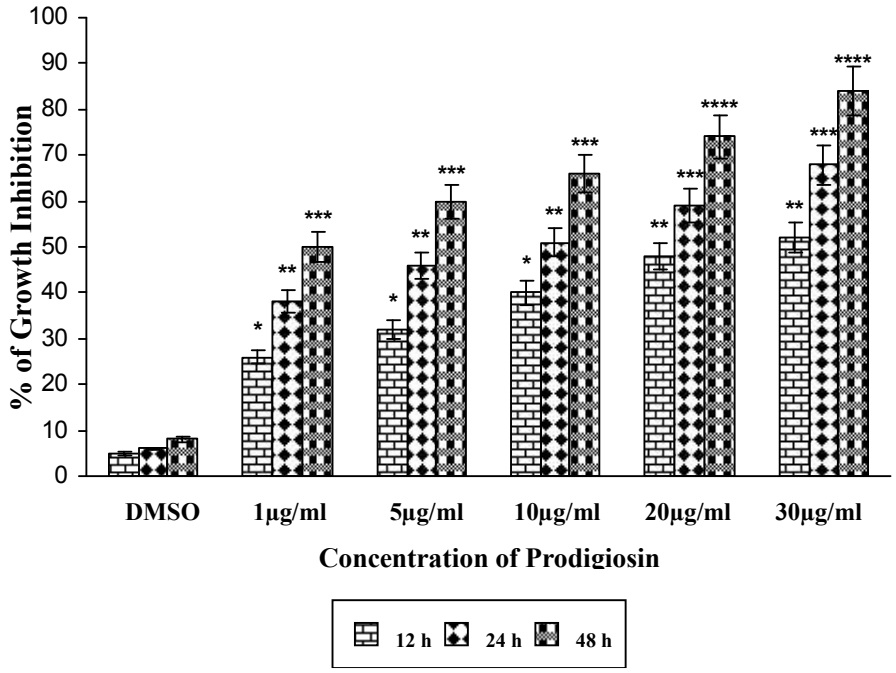

Fig. l. (a) MTT assay showing prodigiosin treatment for 12, 24 and 48 h dose-dependently inhibited population growth of HSC-2 cells.

Assessment of cell injury

HSC-2 cells were cultured with a range of concentration of prodigiosin (1–10 µg) for up to 48 h, and release of LDH (an indicator of membrane integrity) was measured. Results indicated that increase of LDH release of HSC-2 cells took on both time and concentration characteristic (Fig.3).

Effects of prodigiosin induced apoptosis in HSC-2 cells

Fig. 2 (a) shows by fluorescence microscopy morphological changes in DMSO and prodigiosin-treated HSC-2 cells after staining with Hoechst 33258 staining, with heterogeneous intensity and chromatin condensation being apparent under fluorescence microscope. Normal cells were seen as round-shaped nuclei with a homogeneous fluorescence intensity. Treatment with 1, 5, and 10 µg of prodigiosin significantly protected the cells from the morphological changes in HSC-2 cell at 24h incubation. Fig. 2 (b) reveal agarose gel electrophoretic pattern of nuclear DNA from control and prodigiosin-treated HSC-2 cells. Prodigiosin 24 h treatment resulted in apoptosis, as evidenced by specific shearing of DNA, which is considered to be the hallmark of apoptosis.

Changes in the levels of caspase-3 and 9 in HSC-2 cells

Fig. 4 shows the changes in the levels of caspase-3 and 9 in normal and prodigiosin treated HSC-2 cells. There was a significant increase in the levels of caspase 3 and 9 in different doses and time of HSC-2 cells. All the treated cells with prodigiosin showed a significant decrease in the levels of caspase-3 and 9 in DMSO treated cells, but it was more significant in the case of 10 µg of prodigiosin treatment at different hours of incubation period.

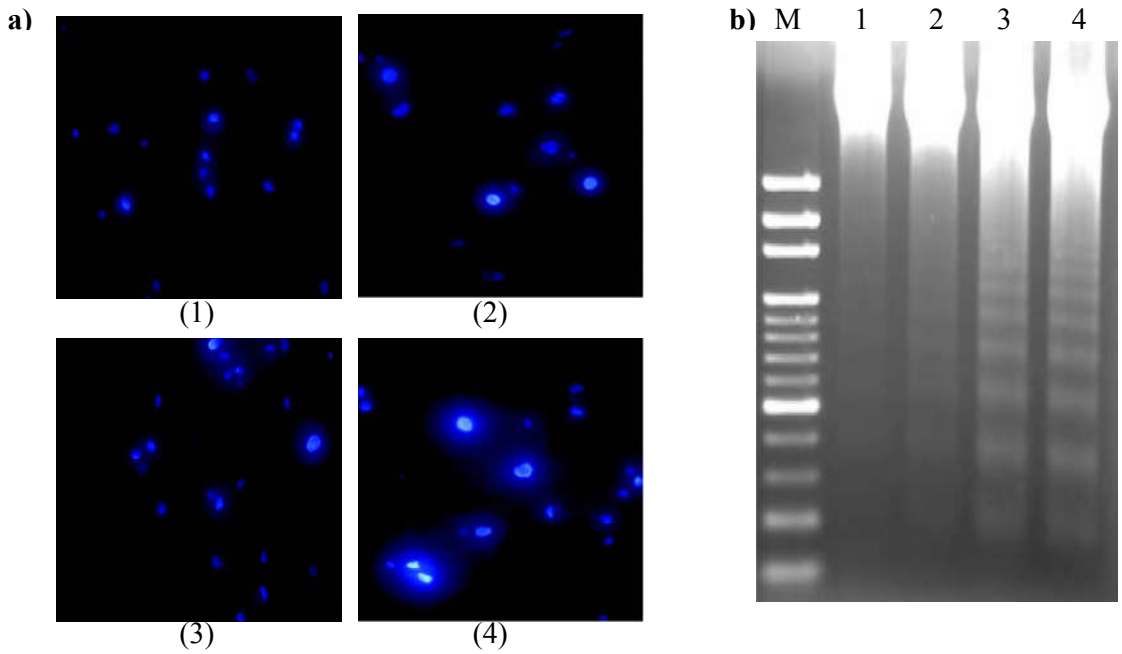

Fig. 2. (a) Silymarin increased percentage of apoptosis in HSC-2 cells as viewed by fluorescence microscopy (Hoechst 33258 staining,×400). (1) DMSO control cells, (2) cells incubated with 1µg/ ml. (3) cells incubated with 5µg/ ml.(3) cells incubated with 10µg/ ml prodigiosin, respectively, for 24 h.(b) Prodigiosin-induced apoptosis in HSC-2 cells as revealed by agarose gel electrophoresis pattern of nuclear DNA. Lanes 1, 2, 3 and M representing HSC-2 control, 1µg/ml, 5 µg/ml and 10µg/ml prodigiosin and marker, respectively for 24h.

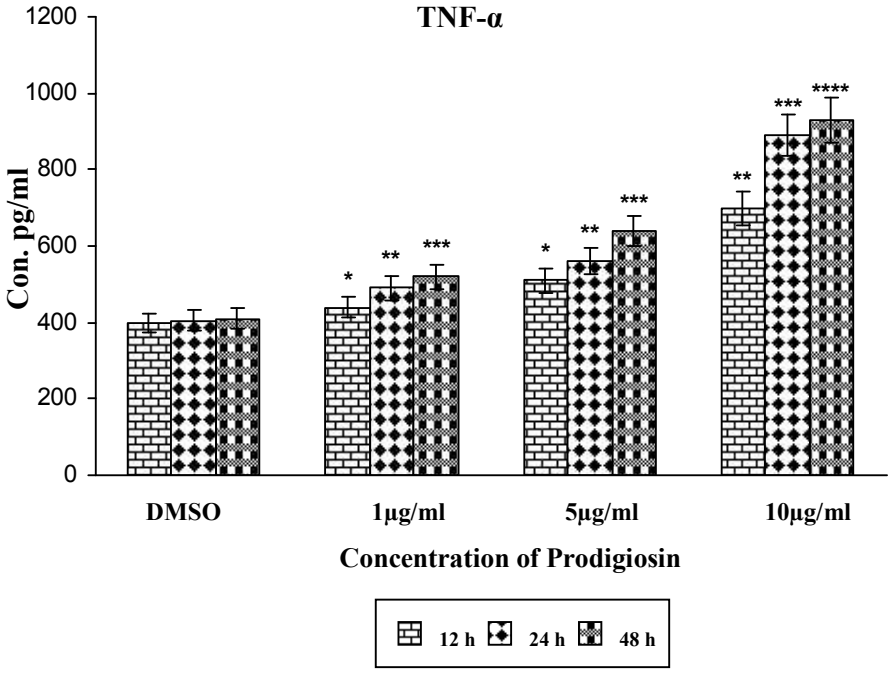

Fig. 3. Time and dose dependent effect of prodigiosin on TNF-alpha secretion in HSC-2

* **Significantly different from control p<0.001 ANOVA followed by DMRT.**
** **Significantly different from control p< 0.05 ANOVA followed by DMRT**
*** **Significantly different form 1,5 and 10µg/ml prodigiosin treated group in 12h incuation time (0.001) ANOVA followed by DMRT**
**** **Significantly different from 24 h same prodigiosin dose ($p < 0.005$) ANOVA by followed by DMRT**

DISCUSSION

In the present study, induction of apoptosis by prodigiosin in human oral cancer HSC-2 cells was assessed by procedures, such as H33258 and DNA fragmentation. All the above clearly indicate that prodigiosin induced apoptosis. Cell death is as important as cell proliferation in regulating development and homeostasis in multicellular organisms (Arend *et al.,* 1990, Barres *et al.,* 1992). Physiological cell death is usually mediated through apoptosis, which is positively or negatively

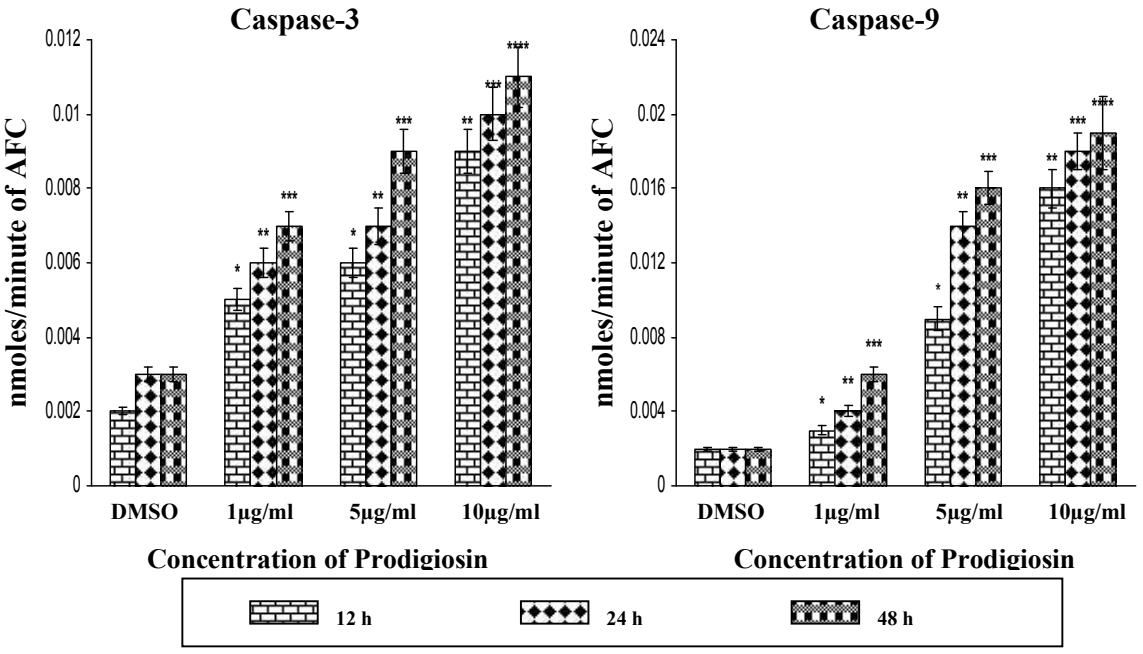

Fig. 4.Time and dose dependent effect of prodigiosin on caspase-3 and 9 activity in HSC-2 cells

* **Significantly different from control p<0.001 ANOVA followed by DMRT.**

** **Significantly different from control p< 0.05 ANOVA followed by DMRT**

*** **Significantly different form 1,5 and 10µg/ml prodigiosin treated group in 12h incuation time (p<0.001) ANOVA followed by DMRT**

**** **Significantly different from 24 h same prodigiosin dose ($p < 0.005$) ANOVA by followed by DMRT**

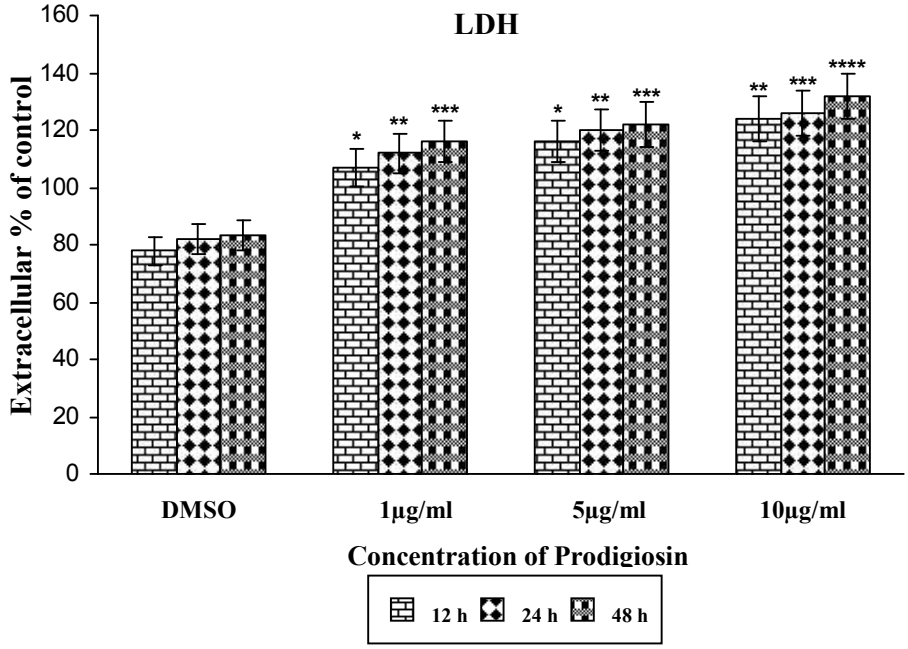

Fig. 5. Time and concentration dependence of prodigiosin-induced LDH release in HSC-2 cells. HSC-2 cells were incubated for various times up to 48 h with up to 10µg/ml. At indicated time, release of LDH was measured as described in Section 2. All data were expressed as mean±S.D. ($n = 3$).

* **Significantly different from control p<0.001 ANOVA followed by DMRT.**

** **Significantly different from control p< 0.05 ANOVA followed by DMRT**

*** **Significantly different form 1,5 and 10µg/ml prodigiosin treated group in 12h incuation time (p<0.001) ANOVA followed by DMRT**

**** **Significantly different from 24 h same prodigiosin dose ($p < 0.005$) ANOVA by followed by DMRT**

regulated by various extracellular factors. It has been demonstrated that a wide range of anticancer agents induce apoptosis in malignant cells in vitro (Uchikawa *et al.,* 1996). Apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, and the activation of tumour necrosis factors (TNF).

TNF-α triggers apoptosis via a Fas-associated protein that activates the death domain and caspase cascade leading to cell death (Taub, 1996). TNFR1 also activates transcription factors, such as NF-kB, which promotes cell growth and differentiation (Heller and Kronke, 1994; Hotamisligil and Spiegelman, 1994). The present data has demonstrated a causal role of TNF- α in apoptosis. Our time related exposure experiment showed marked induction of TNF- α and secretion in cell supernatant as compared to control cells and also time dependent increase in level of the same. Present results shows that prodigiosin is a strong inducer of apoptosis in HSC-2 cells, caspase- 3 and 9 activity which was induced dose dependently and time dependently in comparison with prodigiosin and DMSO treated cells. These observations were crucial as the regulation of cell cycle and apoptotic machinery are important in the growth and development of neoplasms, checkpoint signals, which may result in an activation of pathways leading to program cell death (Sasagawa *et al.,* 2000). It is seen in our study that LDH is induced significantly lower in 48 h groups, however, no significant induction was observed in 12 h study and these results also give some insight about apoptotic activity of prodigiosin in HSC-2 cells. However, Microbial pathogens engage or circumvent the host apoptotic programme. Indeed, PGs have been shown to induce apoptosis this can be corroborated with previous results with various cancer cell lines. (Solé *et al.,* 1994, Montaner *et al.,* 2001, Pandey *et al.,* 2009, Ho *et al.,* 2009). Collectively, evidences suggest that prodigiosin may be used as a potential anticancer agent, although the relevance of the observation to cancer prevention and therapy remains to be elucidated.

In recent years new interest in new drugs associated with apoptosis are expected to be most effective against tumours with high proliferation rates and are being screened for use in the treatment of cancer. In our report, the results suggests that prodigiosin (secondary metaoblites from microorganism) have the apoptotic efficacy as it potently induces caspases 3 and 9 but inhibit TNF-α either directly or through other mechanisms. Furthermore, our study demonstrates that prodigiosin have inhibitory effect in HSC-2 cells and can be used as potent anticancer agents.

REFERENCES

Arend, M.J., Morris, R.G., Wyllie, A.H., 1990. Apoptosis: the role of the endonucleases. Am. J. Pathol. 136, 593–608.

Argiris, A., Karamouzis, MV., Raben, D., Ferris, RL. 2008. Head and neck cancer, *Lancet*, 371, 695-1709.

Ashburner, M., Bonner, J.J. 1979. The induction of gene activity in Drosophila by heat shock. Cell., 17: 241–254.

Barres, B.A., Hart, I.K., Coles, H.S.R., Burne, J.F., Vyodic, J.T., Richardson, W.D., Raff, M.C., 1992. Cell death and control of cell survival in the oligodendrocyte lineage. Cell 70, 31–46.

Cragg, G. M., Grothaus, P. G., and Newman, D. J. 2000. Impact of natural products on developing new anti-cancer agents. *Chem. Rev.,* 109: 3012–3043.

Du, Y., Guo, H., Lou, H., 2007. Grape seed polyphenols protect cardiac cells from apoptosis via induction of endogenous antioxidant enzymes. J. Agric. Food Chem. 55, 1695–1701.

Felthaus, OT., Ettl, M., Gosau, O., Driemel, G., Brockhoff, A., Reck, K., Zeitler, M., Hautmann, TE., Reichert, G., Schmalz, C., Cancer stem cell-like cells from a single cell of oral *Squamous carcinoma* cell Lines. *Biochem. Biophy. Res.,* Comm.DOI:10.1016/j.bbrc.2011. 02.084

Fürstner, A. 2003. Chemistry and biology of roseophilin and the prodigiosin alkaloids: a survey of the last 2500 years, Angew. Chem. Int. Ed Engl., 42, 3582–3603.

Heller, R.A., Kronke, M., 1994. Tumor necrosis factor receptormediated signaling pathways. *J. Cell. Biol.,* 126 (1), 5–9.

Ho, T. F., Peng, Y. T., Chuang, S. M., Lin, S. C., Feng, B. L., Lu, C. H., Yu, W. J., Chang, J. S., and Chang, C. C. 2009.Prodigiosin down-regulates survivin to facilitate paclitaxel sensitization in human breast carcinoma cell lines. *Toxicol. Appl. Pharmacol.,* 235, 253–260.

Hotamisligil, G.S., Spiegelman, B.M., 1994. Tumor necrosis factor alpha: a key component of the obesity–diabetes link. *Diabetes*, 43, 1271–1278.

Kaur, J., Kaur, J., Ralhan, R. 2000. Induction of apoptosis by abrogation of HSP70 expression in human oral cancer cells, Int. J. Cancer., 85: 1–5.

Kim, H.M., Lee,Y.M., 1999. Role of TGF-beta 1 on the IgE-dependent anaphylaxis reaction. *J. Immunol.,* 162, 4960–4965.

Ma, X., Wang, Z. 2009. Anticancer drug discovery in the future: an evolutionary perspective, *Drug Discov. Today.,* 14: 1136–1142.

Montaner, B., Navarro, S., Pique, M., Vilaseca, M., Martinell, M., Giralt, E., 2000. Prodigiosin from the supernatant of Serratia marcescens induces apoptosis in haematopoietic cancer cell lines. *Br J Pharmacol.,* 131: 585–93.

Montaner B, Pe´rez-Toma´s R. 2001. Prodigiosin-induced apoptosis in human colon cancer cells. *Life Sci.,* 68:2025–36.

Pandey, R., Chander, R., Sainis, K. B. 2009. Prodigiosins as anti cancer agents: living upto their name. *Curr. Pharm. Des.,* 15, 732–741.

Papadopoulos, M.C., Koumenis, I.L., Dugan, L.L., 1997.Vulnerability to glucose deprivation injury correlates with glutathione levels in astrocytes. *Brain Res.*, 748, 151–156.

Ravelo, A. G., Estévez-Braun, A., Chávez-Orellana, H., Pérez-Sacau, E., Mesa-Siverio, D.2004. Recent studies on natural products as anticancer agents. *Curr. Top. Med. Chem*., 4, 241–265.

Sasagawa, T., et al., 2000. Synergistic induction of apoptosis in murine hepatoma Hepa1-6 cells by IFN-gamma and TNF-alpha. *Biochem. Biophys. Res. Commun*., 272, 674–680.

Shen, H.M., Ong, C.N., Shi, C.Y., 1995. Involvement of reactive oxygen species in aflatoxin B1-induced cell injury in cultured rat hepatocytes. *Toxicology*, 99, 115–123.

Solé, M., Rius, N., Francia, A., and Lorén, J. G.: The effect of pH on prodigiosin production by non-proliferating cells of *Serratia marcescens. Lett. Appl. Microbiol.,* 19, 341–344 (1994).

Taub, R., 1996. Liver regeneration in health and disease. Clin. Lab. Med. 16 (2), 341–360.

Uchikawa, O., Fukatsu, K., Suno, M., Aono, T., Doi, T. 1996. In vivo biological activity of antioxidative aminothiazole derivatives. *Chem. Pharm. Bull.,* 44: 2070–2077.

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