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In Vitro Inhibition of Growth and Induction of Apoptosis by Mosinone-A in HEp-2 Oral Cancer Cells

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Abstract: The present study evaluated the influence of a Mosinone-A on the induction of apoptosis in HEp-2 human pharyngeal carcinoma cell lines as evidenced by cytotoxicity morphological changes, inhibition of cell proliferation in a time and dose dependent manner. Oral cancer is one of the leading causes of cancer worldwide. Many chemotherapeutics from plants have been tested in cancer, such as Mosinone-A, isolated from the bark of *Annona squamous*, acetogenin. further we analyzed anticancer activity by MTT assay, the generation of reactive oxygen species (ROS), the level of mitochondrial membrane potential and apoptotic morphological changes were analyzed by AO/EtBr. The results of an MTT assay demonstrated that Mosinone-A inhibited the growth of HEp2 cells in a dose-dependent manner. In addition, the induction of apoptosis was detected, as determined by morphological observation. These results indicated that Mosinone-A may induce apoptosis of HEp2 cells through the mitochondrial pathway.

Key words: HEp-2, cell line, MTT assay, MMP, apoptosis, chemoprevention

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1. INTRODUCTION

Oral squamous cell carcinomas are the fourth most common malignancy of the oral cavity, representing 90–95% malignancies in the oral region. The development of oral carcinoma proceeds through a series of genetic changes involving the activation of oncogenes and loss of tumor suppressor genes. [1] and the treatment of oral cancer includes radiation, surgery and chemotherapy.[2] The incidence of oral cancer remains high in Asian and Western countries and Taiwan. The induction of oral carcinoma is preceded by the occurrence of oral cellular damage via reactive oxygen species (ROS) and the generation of chronic inflammation related to oral carcinogenesis. Reactive oxygen species (ROS) may induce mitochondrial depolarization and consequently induce apoptosis in cells [3]. It has been reported that oxidant or oxidant-promoting agents cause necrosis at high concentrations and apoptosis at lower concentrations[4]. Recent studies have demonstrated that ROS and the resulting oxidative stress play a pivotal role in apoptosis [5]. Alternate search for different modes of chemoprevention which are more effective in preventing and reducing the risk of cancer growth is consistently gaining immense focus [6]. The major activities related to the chemopreventive properties of Mosinone-A have been reported to result from inhibition of activity of many protein kinases, blockage of the activation protein-1 (AP-1) and nuclear factor, inhibition of cell proliferation, induction of apoptosis, modulation of cell cycle regulation, interference of receptor binding, and suppression of invasiveness and angiogenesis and undifferentiated oral cancer cell [7] .Mosinone-A is one of the novel mono-tetrahydrofuran ring acetogenin, from the bark of Annona squamosa, viewing cytotoxic selectivities for the human pancreatic carcinoma cell line[8] However, To our knowledge there is no scientific reports were available on the literature for in vitro inhibition of growth and induction of apoptosis in oral cancer cell line by Mosinone-A. Therefore, in the present study, we evaluated the effect of Mosinone-A on induced apoptosis in HEp-2 tumor cells such as typical apoptotic features, formation of apoptotic bodies and reactive oxygen species (ROS) production.

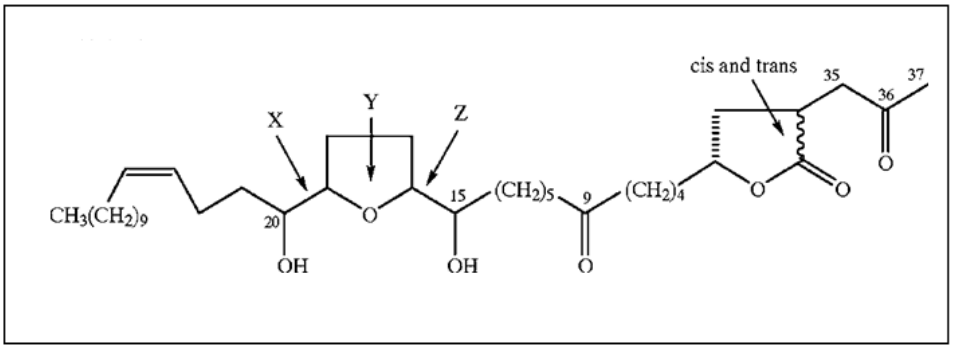

Fig 1: Structure of Mosinone-A (C37H64O7)

2. MATERIALS AND METHODS

2.1. Mosinone-A

Mosinone-A was isolated from *Annona squamosa* bark by the method of Maclaughlin and Hoop (2004). The dried and pulverized bark of *Annona squamosa* was extracted with ethanol. The residues were portioned between chloroform and water. Further portioned between 90% methanol and hexane to got hexane soluble residues, then the hexane soluble residue was subjected into column chromatography over silica gel using hexane and chloroform, then chloroform and methanol as a solvent system. The fractions were combined on the basis of HPTLC. Then the combined fractions were run into column chromatography to get the final product of Mosinone-A. The identify of isolated Mosinone-A was done by LC-MS and NMR. The isolated Mosinone-A was compared with the reference Mosinone-A purchased from Lock chemicals, china. The yield and purity of the isolated Mosinone-A was found to be 0.21% and >90% respectively. For experimental Mosinone-A was first dissolved in 0.5% DMSO. .[9]

2.2. Cell culture

The Human Laryngeal carcinoma (HEp-2) cell line was purchased from NCCS, Pune, India. Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) and maintained at 37 C in a humidified atmosphere containing 5% CO2 and 95% air incubation. Mosinone-A freshly dissolved in 1% dimethyl sulfoxide (DMSO) before treatment.

2.3. Antiproliferation Assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) Assay, HEp-2 oral cancer cells were seeded in 96 well microtiter plate (5×10^3 cells/well) and incubated for 24 hr. The cells were incubated with Mosinone-A at different concentrations ranging from 5-50µM of Mosinone-A for 24 hr. The untreated cells serve as control. MTT solution (5 mg/mL) was added to each well in the 96 well plate and formazan blue color was allowed to develop for additional 4 hr incubation. An equal volume of DMSO was added to stop the reaction and to solubilize the blue crystals. Samples were transferred into culture plates and the absorbance was measured colorimetrically at 590 nm.

2.4. Assessment of Apoptotic and Morphological changes by Acridine Orange/ Ethidium Bromide (Dual Staining Method)

The HEp-2 cells were grown in 6-well plates (5×10^3) for 24 hr, then treated with IC_{50} concentration and 50 µM concentration of Mosinone-A were incubated in CO_2 incubator for 24 and 48 hr. The medium was discarded and the cells were washed in PBS. The cells were trypsinized and stained with a 1:1 ratio of AO/EBr. Stained cells were immediately washed again with PBS and viewed under a

fluorescence microscope with a magnification of 40x (Nikon, Japan).

2.5. *Effect of mitochondrial transmembrane potential*

The cells 1x10^6 cells/mL were cultured in a 6-well plate and treated with IC_{50} and 50µM concentration of Mosinone-A for 24 hrs and the untreated cells served as control. The cells were then stained with Rhodamine 123 (Rh 123) dye (10 µg/mL) and the cells were kept incubated for 30 minutes in a CO2 incubator. The cells were washed by the addition of warm PBS and the mitochondrial depolarization patterns of the cells were observed in the fluorescence microscope (Carl Zeiss, Jena, Germany) using a blue filter.

3. RESULTS

3.1. *Cytotoxic effect of Mosinone-A on HEp-2 cells*

The cytotoxic effect of Mosinone-A was examined on cultured HEp-2 oral cancer cells by exposing the cells to 5-50µM concentration for 24 and 48 hr. MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay, in which the yellow tetrazolium salt is metabolized by NAD-dependent dehydrogenase (in active mitochondria) to form a dark (violet) blue formazan product and the absorbance is directly proportional to the number of viable cells. The reduced MTT-formazan was dissolved in DMSO and the absorbance was read in colorimetry. The graph was plotted as the percentage of inhibition on (X-axis) against the concentration of Mosinone-A (Y-axis). The IC_{50} value was determined by the concentration of the drug which reduces the absorbance to half that of the control. Mosinone-A inhibitor induces cancer cell death in a dose and time-dependent manner. The IC_{50} values of HEp-2 cell line are shown in Figure2.

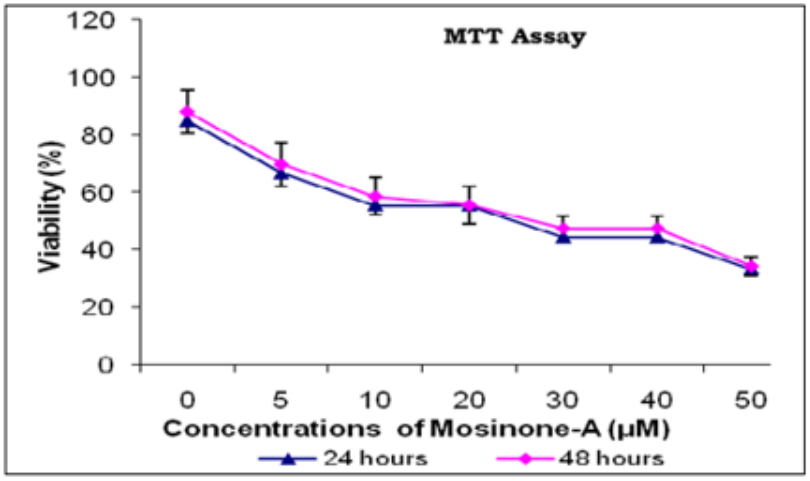

Fig 2: Cytotoxic effect of Mosinone-A on HEp-2 cells

Mosinone-A induced apoptosis in HEp-2 cancer cells. HEp-2 cells were exposed to Mosinone-A at various doses (5-50 µM). The antiproliferative effect was measured by MTT assay and IC_{50} values were calculated (20 µM for 24 and 48hrs). The values are expressed as mean ± SD from the six independent experiments.

3.2. *Effect of Mosinone-A on intracellular ROS of HEp-2 cells*

2, 7, -diacetyl dichlorofluorescein (DCFH-DA) is readily taken up in the cells, hydrolyzed by cellular esterase to DCF, in turn, reacts with ROS and produce fluorescence DCF. The levels of ROS were measured by using a non-fluorescent probe, 2, 7, -diacetyl dichlorofluorescein (DCFH-DA) that can penetrate into the intercellular matrix of cells where it is hydrolyzed by cellular esterases to form dichlorofluorescein (DCFH). HEp-2 cells treated with IC_{50} concentration of Mosinone-A for 24 hr. The intracellular ROS level of HEp-2 cells increased as compared to the control (or) untreated cells. The Levels of ROS in control and Mosinone-A treated cell were depicted in the Figure3. Mosinone-A treatment significantly increases ROS levels in HEp-2 cells. Among the doses tested the IC_{50} value shows 50% of HEp-2 cells in 24 hrs incubation shown in the Figure3 and the percentage of ROS were shown in Figure 3

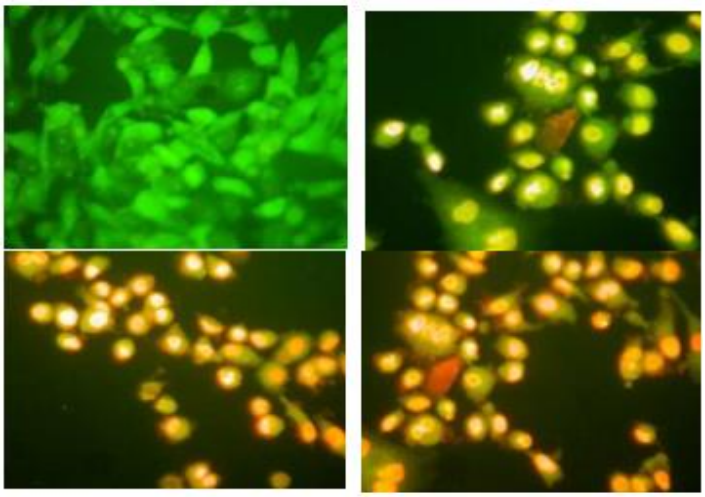

Fig 3: The effect of Mosinone-A on intracellular ROS in Mosinone-A treated HEp-2 cells in 24 hrs a. Morphological changes in the cells, as an indicator of the nature of cell death

Acridine orange (AO)- Ethidium bromide (EB) staining was done on HEp-2 oral cancer cells treated with IC_{50} of 20µM concentration of Mosinone-A for 24 hr. The changes determined by the color variation of cells. The control or untreated cancer cells appeared green in color but the Mosinone-A treated cancer cells for 24 and 48 hr revealed changes. Acridine orange is a cationic dye that enters only live cells and stains DNA and hence the live cells are observed as green under blue emission. Ethidium bromide stains DNA in the cells undergoing apoptosis and hence apoptotic cells appeared orange in color and necrotic cells as red in colour. Mosinone-A treated tumor cells characterized by the membrane blebbing chromatin condensation of innumerable micronuclei in cells, thus showing the apoptotic features. The stained HEp-2 cells with IC_{50} concentration of Mosinone-A for 24 showed gradual increase in the apoptotic death (orange), whereas necrotic (red) cells were observed at the 40 µM concentration of Mosinone-A were shown in the Figure 4.

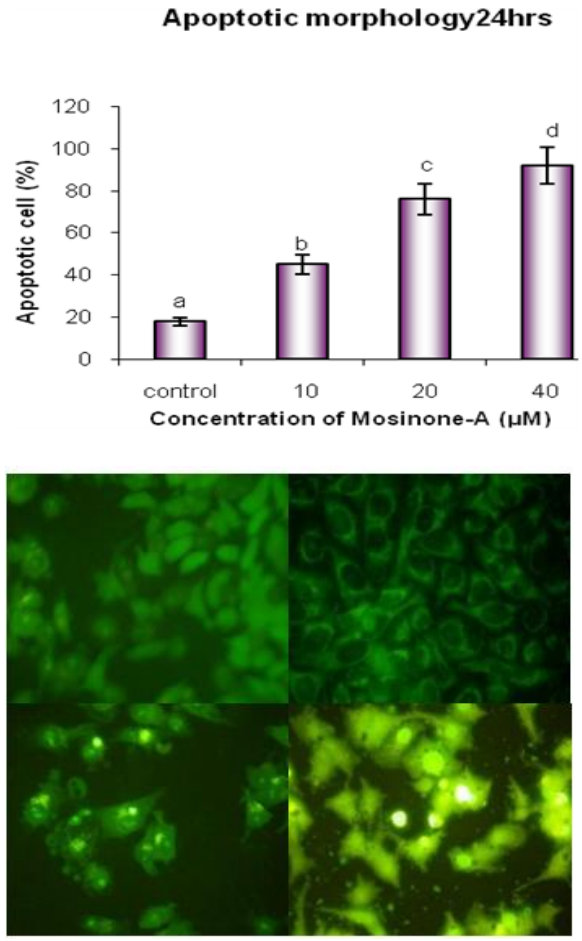

Fig 4: The effect of Mosinone-A on intracellular ROS in Mosinone-A treated HEp-2 cells in 24 hrs.

3.3. *Effect of Mosinone-A on mitochondrial membrane potential by HEp-2 cells*

Mitochondrial swelling is often associated with the loss of mitochondrial membrane potential, measured by using the mitochondrial dye Rhodamine 123 (Rh 123). The HEp-2 cells were treated with IC_{50} concentration of Mosinone-A for 24 hrs, and stained with Rh 123. The results show a significant increase in the progressive loss of red Rh 123 aggregates fluorescence by increasing the incubation time and the concentration of Mosinone-A determines the significant increase in the cytoplasm diffusion of green monomer fluorescence. At the 20 µM concentration of Mosinone-A in both 24 hrs incubation shows complete loss of red fluorescence of Rh 123 and presence of only green fluorescence, where as in the IC_{50} concentration partial or 50% loss of red fluorescence were observed, due to loss of mitochondrial trans-membrane potential in the cancer cells were shown in Figure at the 40 µM concentration of Mosinone-A were shown in the Figure 4and percentage of apoptosis were shown in Figure 5.

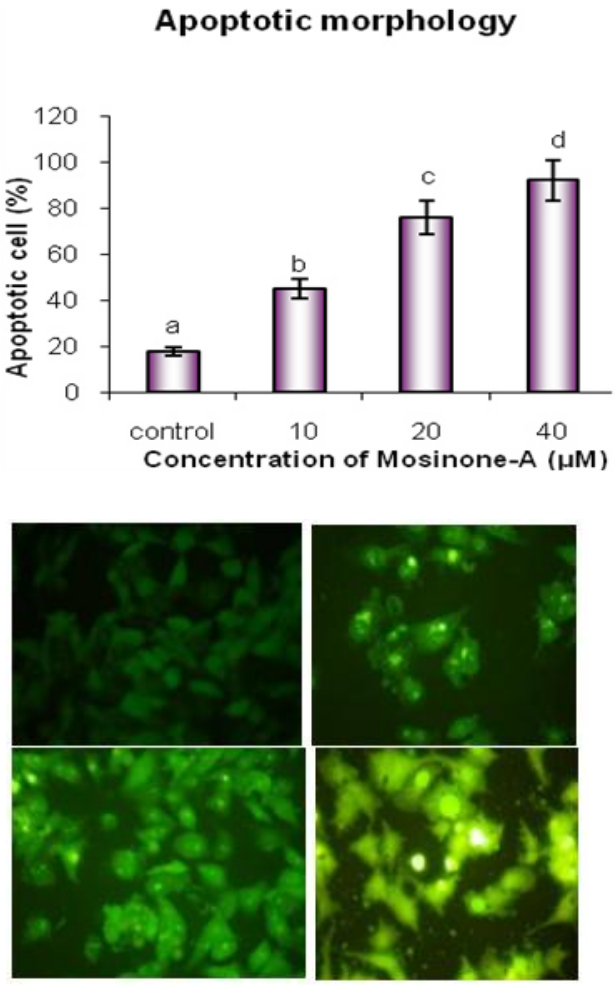

Fig 5: The effect of Mosinone-A on mitochondrial membrane potential in HEp-2 cells in 24 hrs

4. DISCUSSION

The present study demonstrates the Mosinone-A exerts cytotoxic effects against the human oral cancer cell line (HEp-2) in a dose dependent manner with the IC_{50} values of 20µM was determined. The compound which induces apoptosis and significantly inhibited the cell proliferation. [9,] Mosinone-A showed significant activities against various cancerous cell lines, but still the mechanism of these activities have not been fully examined. In this present study, we have investigated the activities of Mosinone-A against the oral cancer cell line HEp-2 by adapting various methods with different doses of Mosinone-A.[10] Methods involved such as cytotoxicity, by analyzing the mitochondrial membrane potential, changes in intra cellular, apoptosis. The present study deals with the early cellular response induced by Mosinone-A treatment in an attempt to define the events involved in cytotoxicity. Profound studies on chemoprevention of human pancreatic and prostate carcinoma cell line offers Mosinone-A as chemo preventive agent due to its diverse pharmacological properties. [11,12], It has been pointed out Mosinone-A has a role to play in the initiation of cellular differentiation, apoptosis, and inhibition of cell proliferation and modification of cell cycle progression. [13.] Apoptosis is the process of cell death characterized by the cellular rounding-up and nuclear fragmentation [14, 15.] Mosinone-A is more potent to inhibit cell proliferation and induce apoptotic cell death. The results of the present study clearly illustrated that Mosinone-A induces cell death through apoptosis by morphological changes in the cells, changes in the intracellular ROS level, loss of mitochondrial membrane potential and cell cycle arrest. Mitochondrial transmembrane potential is a key role in both necrotic and apoptotic cell death [16.] Mitochondria are well known as targets for chemotherapeutic agents because they have a central role in the induction and regulation of apoptotic cell death [17.] Our

data also revealed that Mosinone-A induced the loss of mitochondrial membrane potential, by the mitochondrial dysfunction leading to the cell viability loss and mitochondrial mediated cell death. The data of the present study showed the effect of Mosinone-A on the intracellular ROS by increasing its level in the cell; it is possible that considerable increase in intracellular ROS level enhances the cell death by the treatment of Mosinone-A. Mosinone-A suppresses the growth of the HEp-2 cells and inhibits cell division by the mechanism of cytotoxicity. Apoptotic cell death was shown initially with the lower concentration of Mosinone-A. However, at the higher concentrations and long incubation time of Mosinone-A necrotic cell death was also observed. The necrotic cell death occurred as a process of secondary event, it is a phenomenon seen in vitro due the lack of phagocytosis. Mosinone-A can induce both forms of cell death (apoptotic necrotic cell death). Mosinone-A produce dose and time dependent cytotoxic effect were observed in MTT assay and it was found to induce in HEp-2 cells. The results revealed apoptotic cell death characterized by chromatin condensation, nuclear fragmentation and formation of apoptotic bodies observed in AO/EB staining.

5. LIMITATION

The research work was very effectiveness of the compounds to the cancer cell line because cell line misidentification and contamination with microorganism such as mycoplasma, together with instability both genetic and phenotypic are among the problem that continue to affect the cell culture.

6. CONCLUSION

Thus, this study shows that the Mosinone-A has cytotoxicity effects on HEp-2 oral cancer cells. By inhibiting the growth of the cells, trigger effects on apoptotic cell death and cell cycle arrest. The results of the present study suggest that Mosinone-A can be a promising anticancer therapeutic agent for oral cancer while chemotherapy treatment by Mosinone-A prevents damages of non-tumor tissues. These observations about the apoptotic effects of Mosinone-A on oral cancer cells may be considered as a good approach in research and development in the use of new anti-oral cancer drugs.

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8. AUTHORS CONTRIBUTION STATEMENT

Dr. K. Suresh conceptualized, designed and gathered data, Dr. G. Sugunadevi worked to the manuscript, collection of references as well as interpretation of results.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

10. REFERENCES

1. Kupferman ME, Myers JN. Molecular Biology of oral cavity squamous cell carcinoma. Otolaryngol Clin North Am. 2006;39(2):229-47. doi: 10.1016/j.otc.2005.11.003, PMID 16580909.
2. Parkin DM, Läärä E, Muir CS. Estimates of the worldwide frequency of sixteen major cancers in 1980. Int J Cancer. 1988;41(2):184-97. doi: 10.1002/ijc.2910410205.
3. Chen C, Kong AN. Dietary cancer-chemopreventive compounds: from signaling and gene expression to pharmacological effects. Trends Pharmacol Sci. 2005;26(6):318-26. doi: 10.1016/j.tips.2005.04.004, PMID 15925707.
4. Hopp DC, Zeng L, Gu Z, McLaughlin JL. Squamotacin: an annonaceous acetogenin with cytotoxic selectivity for the human prostate tumor cell line (PC-3). J Nat Prod. 1996;59(2):97-9. doi: 10.1021/np960124i, PMID 8991957.
5. Sankaranarayanan R, Ramadas K, Thomas G, Muwonge R, Thara S, Mathew B, et al. Effect of screening on oral cancer mortality in Kerala, India: a cluster-randomised controlled trial. Lancet. 2005;365(9475):1927-33. doi: 10.1016/S0140-6736(05)66658-5, PMID 15936419.
6. De Felice F, de Vincentiis M, Valentini V, Musio D, Mezi S, Lo Mele L; et al. Follow-up program in head and neck cancer. Crit Rev Oncol Hematol. 2017;113:151-5. doi: 10.1016/j.critrevonc.2017.03.012, PMID 28427504.
7. Essa AAM, Deraz EM. Expression of CD44 (NKI-P1) in oral squamous cell carcinoma associated vascular endothelial cells: a relationship to tumor angiogenesis. Saudi Dent J. 2022;34(1):21-6. doi: 10.1016/j.sdentj.2021.09.022. PMID 35068895.
8. Hopp DC, Zeng L, Gu ZM, Kozlowski JF, McLaughlin JL. Novel mono-tetrahydrofuran ring acetogenins, from the bark of Annona squamosa, showing cytotoxic selectivities for the human pancreatic carcinoma cell line, PACA-2. J Nat Prod. 1997;60(6):581-86. doi: 10.1021/np9701283, PMID 9214729.
9. Zeng L, Wu F, Oberlies NH, McLaughlin JL, Sastrodihadjo S. Five New Monotetrahydrofuran Ring Acetogenins from the Leaves of Annona muricata. J Nat Prod. 1996;59(11):1035-42. doi: 10.1021/np960447e.
10. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. 1997;275(5303):1129-32. doi: 10.1126/science.275.5303.1129, PMID 9027314.
11. Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell. 2006;9(5):351-65. doi: 10.1016/j.ccr.2006.03.027, PMID 16697956.
12. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell. 2005;17(3):393-403. doi: 10.1016/j.molcel.2004.12.030, PMID 15694340.
13. Sadhukhan R, Leone JW, Lull J, Wang Z, Kletzien RF, Heinrikson RL et al. An efficient method to express

and refold a truncated human procaspase-9: a caspase with activity toward Glu-X bonds. Protein Expr Purif. 2006;46(2):299-308. doi: 10.1016/j.pep.2005.09.006, PMID 16256367.

14. Ni Chonghaile T, Sarosiek KA, Vo TT, Ryan JA, Tammareddi A, Moore Vdel G et al. Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. Science. 2011;334(6059):1129-33. doi: 10.1126/science.1206727, PMID 22033517.
15. K L King[1], J A Cidlowski, Cell cycle regulation and apoptosis. Annu Rev Physiol 1998;60:601-17
16. Chaudhry GE, Akim AM, Sung YY, Muhammad TST. Cancer and Apoptosis Methods Mol Biol. 2022;2543:191-210. doi: 10.1007/978-1-0716-2553-8_16.
17. Wang Q, Jiang H, Fan Y, Huang X, Shen J, Qi H et al. Phosphorylation of the α-subunit of the eukaryotic initiation factor-2 (eIF2α) alleviates benzo[a]pyrene-7,8-diol-9,10-epoxide induced cell cycle arrest and apoptosis in human cells. Environ Toxicol Pharmacol. 2011;31(1):18-24. doi: 10.1016/j.etap.2010.08.005, PMID 21787665.
18. Rizzolo D, Hanifin C, Chiodo TA. Oral cancer: how to find this hidden killer in 2 minutes. JAAPA. 2007;20(10):42-7. doi: 10.1097/01720610-200710000-00021, PMID 18019328.