ANTICANCER DRUG FROM POLYHERBAL EXTRACT AND THEIR ROLE IN APOPTOSIS

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ABSTRACT

MOLT-4 is a human cell line with the hypertetraploid chromosome number which is a suitable transfection host. In the present study, anticancer mechanism of polyherbal drug was analysed with various methods which includes MMP assay, ETBR/AO staining , ROS assay, comet assay, apoptosis against human blood cancer MOLT-4 cell line. The Former class stage has DNA in it wherein the later stage there is complete degradation of DNA. The round intact shape conveys that there is no damaged DNA. The use of comet assay confirms that the compound 8 drug is able to cause cellular damage to MOLT-4 cells. The ROS production depicts the presence of free radicals stained by fluorescene. The drug interacted with the cells caused free radical formation which further inhibited the cellular function of tumour cells. Treated MOLT-4 cells were subjected to EtBr/AO staining. The antiproliferative effect of the compound 8 sample upon staining showed apoptotic changes. A progressiveincrease in the number of cells was noted in the treated cells compared to the control cells. Apoptosis is the process of cell death.The present study reveals the drug extract induces the apoptosis by morphological changes in the cell. MMP plays a key role in both necrotic and apoptotic cell death.

Keywords*: MOLT-4 cells, comet, EtBr/AO stain, MMP, Free radicals, ROS.*

INTRODUCTION

Evolutionary adaptation by somatic cells is different from adaptation by organisms, it is an important part of cancer biology and needs to be understood. One centrally important application lies in reconciling two major and contrasting patterns in cancer biology, which we refer to as trait “hallmarks” and molecular “snowflakes.” The first of these two patterns to be described was the striking consistency among different types of cancer at the level of cell traits. Although they originate from different tissues and cell types, virtually all cancers consist of cells with the same essential “hallmark” traits (Hanahan and Weinberg 2000, 2011). This quickly became one of the few organizing frameworks to impose order on the bewildering diversity of cancer. The superficially contrasting pattern that has since emerged from molecular analysis is that virtually any molecular category of cancers can, on closer inspection, be broken down into subcategories by looking for, and invariably finding, molecular variations.

To appropriately allocate resources to prevention, screening, diagnosis, treatment, and palliative care and to monitor their effectiveness, it is necessary to have timely information about cancer burden for individual countries. The Global Burden of Disease (GBD) study provides a comprehensive assessment of incidence, mortality, and disability for all major diseases and injuries. Herein, we present detailed results of the GBD 2013 study1 for 28 cancer groups covering cancer incidence, mortality, and disability for 188 countries from 1990 to 2013 for both sexes and different age groups.

The global burden of cancer is growing, particularly in countries of low and middle income. The need to implement effective strategies of primary prevention is urgent. Prevention is crucial but long

term. If WHO's global target of a 25% reduction in deaths from cancer and other non-communicable diseases in people aged 30–69 years is to be achieved by 2025 (referred to as 25 × 25), we will need not only more effective prevention (to reduce incidence) but also more effective health systems (to improve survival) (4). Surveillance of cancer survival is seen as important by national and international agencies, cancer patient advocacy groups, departments of health, politicians, and research agencies. Cancer survival research is being used to formulate cancer control strategies,to prioritise cancer control measures, and to assess both the effectiveness11,12 and cost-effectiveness (5) of those strategies.

These reports provide annual updates on cancer incidence, deaths, and trends of the most common cancers in the United States. In addition, each publication features an in-depth analysis of a selected special topic. This year's report provides a detailed analysis of the incidence of liver and intrahepatic bile duct (liver) cancers and rates of liver cancer and hepatitis C virus (HCV) infectionassociated deaths. Worldwide, liver cancer is the fifth most common cancer among men, the ninth most common cancer among women, and the second most common cause of cancer death for men and women combined (7). Although liver cancer occurs more frequently in less developed regions of the world,18 it is still a significant health outcome in the United States. In 2012, a total of 28,012 persons in the United States (excluding Nevada) were diagnosed with liver cancer (20,207 men and 7805 women), and 22,972 died of this disease (15,563 men and 7409 women). The relative 5-year survival rate for liver cancer is 16.6% (95% confidence interval [CI], 16.3%–16.9%) (8) Forty-three percent of patients with liver cancer are diagnosed at a localized stage, for which the 5-year relative survival rate is 30.5%.20 Those diagnosed at a regional stage (27%) and a distant stage (18%) have 5-year relative survival rates of 10.7% and 3.1%, respectively (9).

The landscape of cancer therapy has changed dramatically over the last few years because of novel precision medicine and immunotherapy approaches, which provide significant benefit to many cancer patients (10). It is becoming increasingly appreciated that targeted drugs, particularly kinase inhibitors, which make up most targeted therapeutics, can have broadly varying target profiles (6). Thus, using multi-targeted compounds with unexplained anticancer activity as research tools to identify previously unrecognized cancer vulnerabilities constitutes an intriguing novel modality for drug development.

The science of apoptosis has been widely studied and has been applied to the development of drugs targeting apoptosis mechanisms. Translation of this science to development of a predictive test for response to chemotherapy, in order to improve outcomes of chemotherapy, has been a goal of translational science and personalized medicine. Prior chemosensitivity/chemoresistance assays which have sought to predict clinical outcomes but which have not been direct measurements of drug induced apoptosis have performed poorly [12]. Recently, in blinded validity trials, a drug-induced apoptosis assay (called the Microculture Kinetic MiCK Assay) was found to correlate with patient outcomes in acute myelocytic leukemia, and ovarian cancer. When the assay was initially studied in a broad utility trial in a heterogeneous patient population with various cancers, physicians who received results of the MiCK assay prior to deciding on chemotherapy were found likely to use the assay (64% usage), and when physicians used the MiCK assay, there was an increase in complete or partial response rates, time-to-progression, and overall survival [13].

Models to study environmental toxicity are a necessary compromise between the control of experimental parameters (through the use of lab-reared substitute species and the setting of a thoroughly controlled exposure scenario) and realism (field or semi-field studies)

An entirely different approach is based on the use of native species, which essentially considers pollution as a complex situation and therefore implies a more holistic interpretation of the real conditions of exposure in the field. This kind of study includes the capture of animals and/or the collection of plants, water or soil samples on the field. This approach allows considering interactions among pollutants and

also homeostasis. Life-term exposure occurs in a natural context, allowing the action of such modulating factors as discontinuous pattern of pollution, reduction of the animal activity or sheltering. Interpretation of the results, on the other hand, may be particularly difficult in face of the many constraints and confounding factors of the natural environment (14).

During metastatic progression, cancer cells detach from the primary tumour into the surrounding extracellular matrix (ECM). A subpopulation of these cells will intravasate into the blood stream or lymphatic vessels, entering the circulation. Due to their size, these disseminated cancer cells will eventually extravasate via transendothelial migration, and a small proportion

Reactive oxygen species (ROS) have important roles in mediating cell proliferation, migration and angiogenesis through the regulation of many key intracellular signalling pathways including Akt, Stat3, and NF-κB. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) are an important source of ROS. NOX2, also known as gp91phox, is a member of the NOX family that is constitutively associated with p22phox in the plasma membrane. The activation of NOX2 involves its interactions with p40phox, p47phox, p67phox and the small GTPase Rac1 [17]. The aim of the present study was to analyse the mechanism of action of anticancer drug in MOLT-4 cells.

Materials and methods

Chemicals required

The Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit was purchased from Thermo scientific (USA), ETBr, low melting agarose, Normal agarose were from Merk, 1X PBS was from Himedia, (India), Mitochondrial membrane potential assay kit was from Sigma, (USA), Total reactive oxygen species (ROS) kit, Invitrogen (USA), 6 well tissue culture plate and wash beaker were from Tarson (India).

Cell culture

MOLT- 4 (human blood cancer cell) cell line were cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), 100 u/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37ºC.

Apoptosis assay

Briefly, 5 x 10^5 cells/ml of MOLT- 4 (human blood cancer cell) cells were seeded into the 24 well tissue culture plate and treated with 135.4 µg/ml of BSM sample in a serum free DMEM medium. The plate was incubated at 37ºC at 5% Co2 incubator for 24 hours. After incubation, 10 µl of Alexa Fluor and 5 µl of PI were added to the wells and mixed gently and incubated for 15 minutes. After incubation 400 µl of 1x Annexin binding buffer was added and mixed gently.

Finally, the plate was centrifuged at 800 rpm for 2 minutes and evaluated immediately within an hour and examined the cells by fluorescence microscope using a fluorescent filter.

Comet assay

Briefly, MOLT- 4 cells were seeded in a six-well plate at a density of 10,000 cells/well and incubated for 24 h at 37°C in a humidified 5% CO2 incubator. The wells were washed with sterile PBS and treated with 3.46 µM/ml of poly herbal formulation in a serum free DMEM medium and incubated for 24 h at 37°C in a humidified 5% CO2 incubator. The cells were harvested by trypsinization in a 1.5 ml tube and comet assay was performed based on the protocol of nadhakumar et al with slight modifications. The microscopic slides were sequentially coated with 200 µL 0.75 % normal melting agarose as the first

layer and 100 µL of 0.5% low melting agarose as the second layer. The next step was to add 20 µL cell suspensions to 60 µL of 0.5% low melting agarose, which was distributed on the slides as the third layer. Then the slides were incubated in cell lysis buffer (2.5 M NaCl, 0.2 M NaOH, 100 mM Na2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% dimethyl sulfoxide, pH =10.0) for overnight at 4°C. After that, the slides were immersed in double distilled water for three times followed by 20 min incubation of unwinding solution (3 M NaOH). Subsequently, the slides were placed in a horizontal gel electrophoresis tank containing electrophoresis solution (1 mM Na2EDTA and 300 mM NaOH, pH =13). The electrophoresis was conducted at 25 V (1 V/cm, 300 mA) for 25 min. Then the slides were incubated in neutralization buffer (0.4 M Tris–HCl, pH =7.5) for 10 min followed by immersion in ultrapure water for three times and air-dry. The cells were stained with 50 µL of ethidium bromide (5 mg/L) and observed under fluorescent microscope.

All steps were carried out under dim light to minimize extra DNA damage.

ETBr /AO staining

Briefly, 5 x 10^5 cells/ml of MOLT- 4 cells were plated to a 24 well tissue culture plate and incubated for 24 hr in a DMEM growth medium. After incubation, the plate was washed with PBS and treated with 3.46 µM/ml of compound poly herbal formulation in a serum free DMEM medium. The plate was incubated at 37ºC at 5% Co2 incubator for 24 hours. After incubation, 50 µl of 1 mg/ml acridine orange and ethidium bromide were added to the wells and mixed gently. Finally, the plate was centrifuged at 800 rpm for 2 minutes and evaluated immediately within an hour and examined at least 100 cells by fluorescence microscope using a fluorescent filter.

Measurement of mitochondrial membrane potential

The MOLT- 4 cells (5,000–20,000 cells/well) were plated to a 24 well plate and incubated for 24 hr in a DMEM growth medium. After incubation, the plate was washed with PBS and treated with 3.46 µM/ml of poly herbal formulation sample in a serum free DMEM medium. Again, the plate was incubated at 37°C in a humidified 5% CO2 incubator for 24 hrs. The measurement of mitochondrial membrane potential for the treated and control cells was carried out according to the manufacturer's instruction. Breifly, the cells were incubated with 100 µl/well of JC-10 dye loading solution and plate was protected from light. The plate was incubated for 30 – 60 minutes in a 5% CO2 at 37 °C. After incubation, 100 µl/well assay buffer B was added to the each sample/well. Finally, the plate was centrifuged at 800 rpm for 2 minutes and the fluorescence was observed at 490/525 and 540/590 ratio.

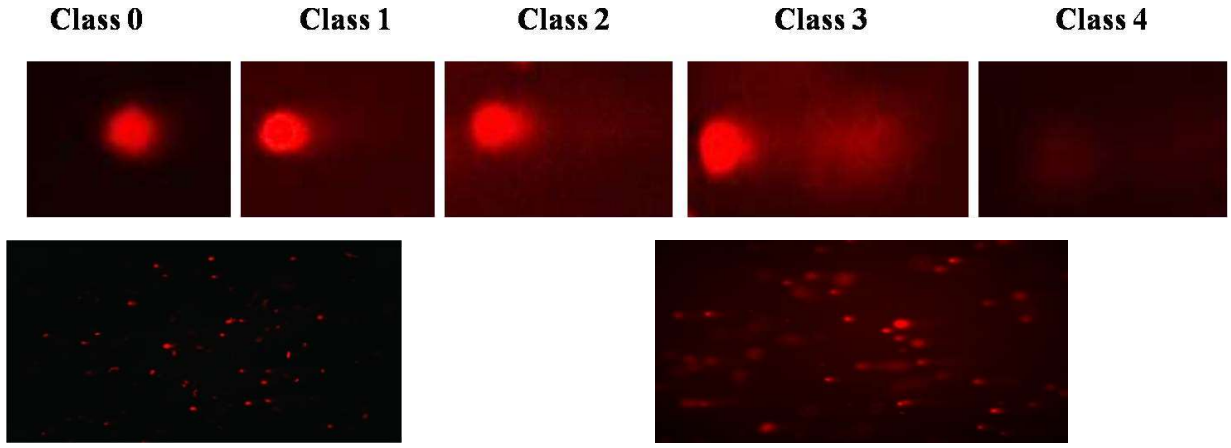
ROS Assay- Total reactive oxygen species assay

The poly herbal formulation was tested for ROS using MOLT-4 cells. Briefly, the cultured Hep-G2 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^6 cells/ml into 6 well tissue culture plate in DMEM medium containing 10 % FBS and 1% antibiotic solution for 24 hour at 37°C. The cells were treated for 3 h with 3.46 µM/ml of 8 sample. Then 1 ml of ROS assay buffer was added followed by 100 µl of 1X ROS assay stain solution was added to the wells and mixed gently. Then the plate was incubated at dark for 60 minutes in a 37ºC incubator with 5% CO_2. After the incubation the production of ROS was evaluated immediately by fluorescence microscope (ZOE, Fluorescent cell Imager, Bio-Rad, USA) using a fluorescent filter at 520 nm.

RESULTS

Comet assay

From the results observed below, the beginning of the class stage has DNA in it wherein the later stage there is complete degradation of DNA. The round intact shape conveys that there is no damaged DNA. The use of comet assay confirms that the drug is able to cause DNA damage to MOLT-4 cells.

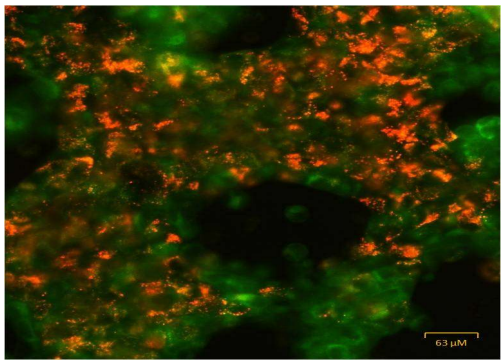


Control **61.91 µg/ml of polyherbal formulation sample**

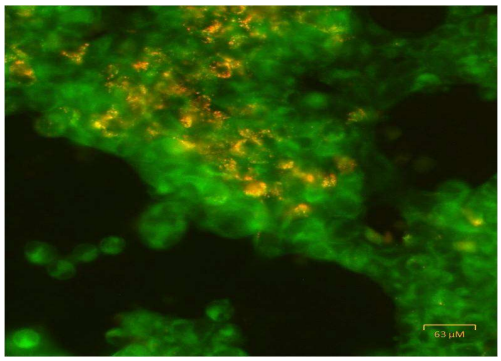
S.No	Class	No.of cells
1	0	4
2	1	7
3	2	6
4	3	5
5	4	9

3.2 MMP ASSAY

The mitochondria being intact and functional could be observed from the healthy control cells. Whereas, the damge is observed in the presence of orange coloured fluorescence produced by JC10 stain. This stain particularly used in mitochondrial membrane.



control

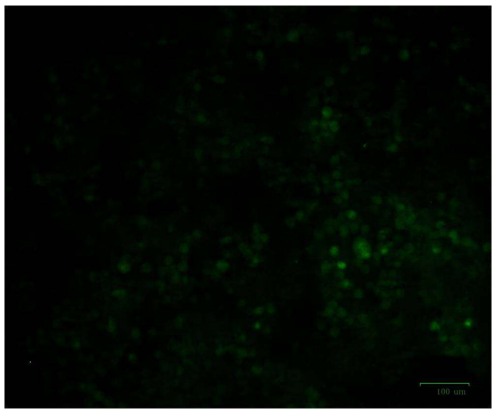


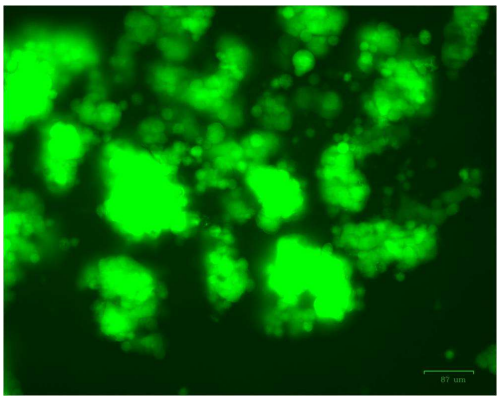
61.91 µg/ml of polyherbal formulation

Control cells

3.3 ROS assay

The reactive oxygen species measures the free radicals formation. The ROS production depicts the presence of free radicals stained by fluorescene. The drug interacted with the cells caused formation of reactive oxygen species which further inhibited the cellular function of tumour cells.

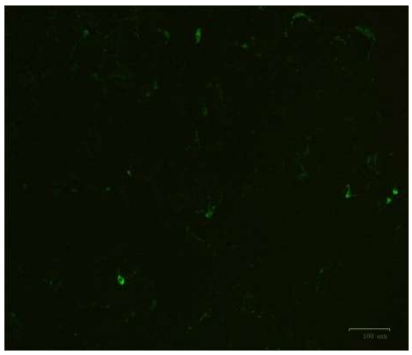


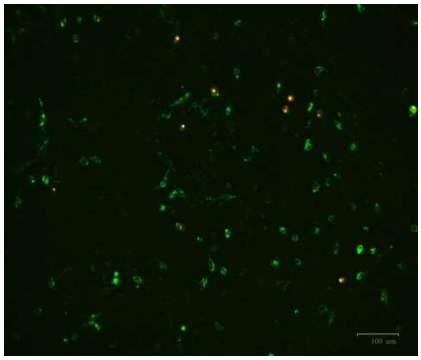


Control **Treated with 61.91 µg/ml of polyherbal formulation sample**

3.4 Apoptosis assay

In the apoptotic treatment, Alexa fluor and PI were the dye being used. The cells been treated showed flourescence stain all over which depicted DNA damage induced apoptosis.

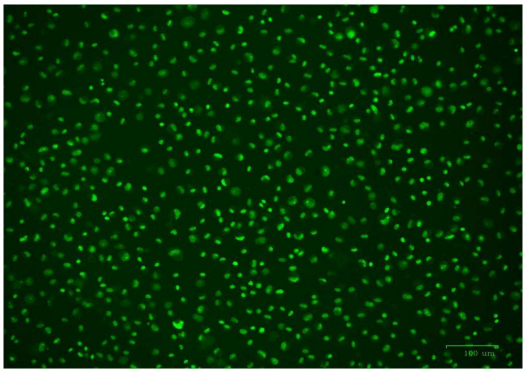


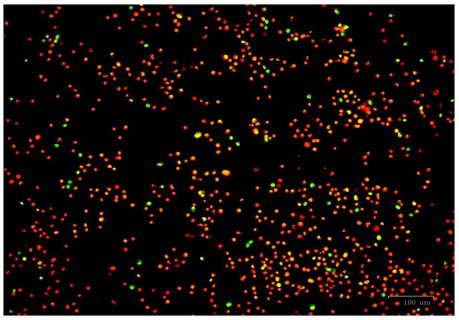


control cells Treated with 61.91 µg/ml of polyherbal formulation sample

4.5. ETBR /AO staining

Polyherbal formulation treated MOLT-4 cells were subjected to AO/EB staining. AO entering the nucleus, stains live cells in green fluorescent colour whereas EB penetrating the dead cells are stained in red due to loss of membrane integrity. The antiproliferative effect of the polyherrbal formulation sample upon staining showed apoptotic changes. A progressiveincrease in the number of cells was noted in the treated cells compared to the control cells.





Control Treated with 61.91 µg/ml of polyherbal formulation sample

Apoptotic and necrotic indexes of compound 8 treated MOLT-4 cells

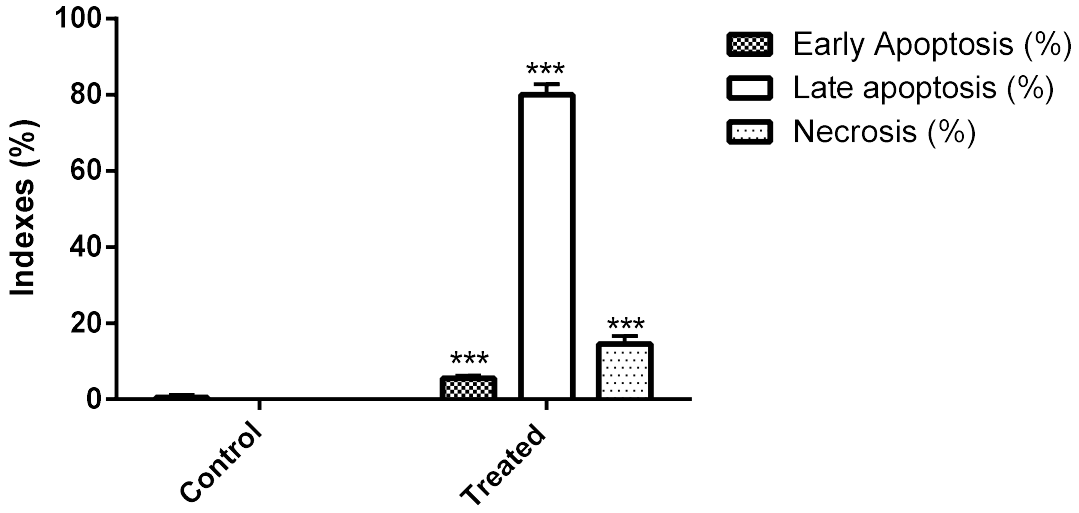


Fig. 1. Bar graph showing the Indexes of eary, late apoptosis and necrosis of control and compound 8 treated MOLT-4 cells.

	Early apoptosis Index (%) (in duplicates)		**Late apoptosis Index (%) (in duplicates)**		**Necrosis Index (%) (in duplicates)**	
Control	1	0	1	2	0	0
Treated with plant extract	6	5	78	82	16	13

(The bar graph was made by calculating 100 cells/Square and their mean values)

Early apoptotic cells – No. of cells which appeared yellow-green fluorescence/100 cells

Late apoptotic cells - No. of cells which appeared orange nuclear fluorescence/100 cells

Necrotic cells- No. of cells which appeared orange-red fluorescence/100 cells

	Early apoptosis Index (%)	**Late apoptosis Index (%)**	**Necrosis Index (%)**
Control	0.5	0	0
Treated	5.5	80	14.5

Table 1. Means of early, late apoptotic and necrotic indexes (%)

Apoptosis is the process of cell death.The present study reveals the extract induces the apoptosis by morphological changes in the cell. MMP is the key role in both necrotic and apoptotic cell death.

5.DISCUSSION

The present study demonstrates mechanism of action of anticancer drug against human blood cancer (MOLT-4) in a dose dependent with the IC50 value of 61.91µg/ml. The compound which induces the apoptosis and inhibits the cell proliferation . Several Methods involved to identify the activities of polyherbal drug against blood cancer cell are mitochondrial membrane potential, ETBR/AO staining , Ros assay, comet assay, and apoptosis.

Cancer is the uncontrolled growth of abnormal cells termed malignant tumor . Cancer is caused by accumulated damage to genes .such changes may be due to chance or to exposure to a cancer causing substance.The substances that cause cancer are called carcinogens . Almost 10 million people die from cancer annually cancer is one of the world's largest health problems .The global burden of disease estimate that 9.56 million people died prematurely as a result of cancer in 2017 .Every six death in the world is due to cancer.

The mechanistic studies discovered that the cell death induced in MOLT-4 cells was autophagic as well as apoptotic in nature. The apoptosis and autophagy induction was confirmed by an array of experiments like cellular and nuclear microscopy, Annexin-V binding, loss of MMP, cell cycle analysis, immunofluorescence and immunoexpression of key apoptotic and autophagic proteins. DNA damage is considered as the sign of apoptosis. [68] MMP is an important role in necrotic and apoptotic cell death.

In conclusion, these results showed that the polyherbal formulation was able to inhibit cancer cell proliferation by inducing apoptosis. Therefore, this polyherbal formulation can be used for the treatment of blood cancer.

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