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Cytotoxic activity of Molt-4, a polyherbal formulation against herbal plants

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ABSTRACT
The Ficus religiosa, Andrographis paniculata, Momordica charantia,Eugenia jambolanaare well-known plants available throughout India and they are commonly used for the treatment of various diseases The polyherbal formulation was formulated using the hydroalcoholic extracts of the Ficus religiosa, , Andrographis paniculata, Momordica charantia, Eugenia jambolanabythe Soxhlet extraction method. The phytochemical characterization was carried out for the polyherbal formulation. Further, the anticancer activity of polyherbal formulation was studied usingMOLT-4cells and their mechanism of action was analyzed by ETBr/AO staining method. The phytochemical characterization results showed the presence of tannins etc. The MTT assay results showed the anticancer activity of polyherbal formulation against MOLT-4 cells and their IC50 value was found to be 61 µg/ml. The ETBr/AO staining results further confirmed the polyherbal formulation. Taken together, these results showed thatthepolyherbal formulation has anti-cancer potential and can be used for the treatment of blood cancer.
Keyword: *anti-cancer, Polyherbal formulation, phytochemical,Soxhlet extraction MOLT-4 cells,IC50,ETBr/AO staining*

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INTRODUCTION

The total research and development (R&D) spending for drug discovery worldwide has increased at least 15-fold from 1975 **.**There are four broad categories of blood cancers: leukaemia, myeloma, Hodgkin lymphoma and non-Hodgkin lymphoma[1]. Together, these account for around 9 % of all cancers and are currently the fourth most common in both males and females in the world**.**But these four categories contain over 60 different subtypes, as first highlighted in 2001, and then in 2008, when the World Health Organisation (WHO) produced an accepted classification of haematologic malignancy that defined each type according to immunophenotype[2], genetic abnormalities and clinical features .Increasing age has been historically implicated in higher mortality after high-dose allogeneic hematopoietic cell transplantation (HCT) for patients with hematologic malignancies. These transplants are preceded by intense, cytotoxic conditioning regimens that are aimed at reducing tumor burden[3]. The risk of organ toxicities has limited the use of high-dose regimens to younger patients in good medical condition. Therefore, age cut-offs of 55–60 years have been in place for decades for high-dose HCT. This excluded the vast majority of patients from allogeneic HCT given that median ages of patients at diagnoses of most hematologic malignancies range from 65–70 years. For decades the diagnosis, classification and management of hematologic neoplasms was based on the clinico-pathological features of these disorders[4]. Major progress in both the therapeutics and our understanding of these diseases did not occur until 1960 when Philadelphia chromosome was found in bone marrow cells of patients with chronic myelogenous leukemia (CML), followed by the identification of the molecular defect by the fusion of BCR and ABL genes in 1982[5-6].Cervical cancer is one of the serious health problems in women. In India alone, more than 70,000 new cases of cervical cancer are reported every year . Most of the cervical cancers are caused by HPV infection and integration of HPV genome into the host cell's genome .

Ficuspumila, a creeping vine like fig plant, is native to South China and Malaysia. Several studies have been performed on the composition of Ficuspumila, and a number of compounds have been identified, such as apigenin, luteolin, rutin, genistein, hesperidin, astragalin, isoquercitrin, and chrysin[7]. Dried stems and leaves of Ficuspumila have been folklorically used in the treatment of rheumatoid arthritis, edema, tonic medicament, throat pain, and postpartum abdominal pain . However, no research has been investigated on the analgesic and anti-inflammatory mechanisms of *Ficus pumila* yet.

Andrographis paniculata is an important medicinal plant and widely used around the world. It belongs to the family Acanthaceae. AP is used as a traditional herbal medicine in Bangladesh, China, Hong Kong, India, Pakistan, Philippines, Malaysia, Indonesia, and Thailand and is ethnobotanically used for the treatment of snake bite, bug bite, diabetes, dysentery, fever, and malaria . In the Unani and Ayurvedic medicines, AP is one of the most used medicinal plants[8-9].

Morinda citrifolia L. (noni) is an example of a plant used as a functional food and has been widely studied due to its apparent beneficial effects on human health. It has been investigated as an alternative in anticancer, antibacterial, and antimicrobial therapies, and in the treatment of esophageal reflux and ulcers in animals[10]. One of the explanations for the medicinal action of noni fruits is that xeronine could modulate the conformation and stability of specific proteins. Heinicke described beneficial effects of noni fruits, such as in menstrual cramps, hypertension, burns, depression, atherosclerosis, digestion, relief for pain, and many others. *Lantana camara Linn.* (family: Verbenaceae), an ornamental shrub, has spread as an intractable weed in many parts of the world. The genus Lantana contains many species that are native to the Americas and Africa, and has become naturalized as a noxious weed in tropical, subtropical, and warm temperate countries. Lantana camara has been found in nearly 50 countries and is the principal weed in 12 countries. It is a serious weed spreading over Australia, Asia, Africa, South America, and North America. Strategies for the control of lantana have been reviewed in a recent monograph by Day and co-workers . In the present study was to prepare a polyherbal formulation and phytochemical characterization for the treatment of human blood cancer and their mechanism of action by ETBr/AO staining method.

MATERIAL AND METHODS

Chemicals and reagents

DMEM medium, Fetal Bovine Serum (FBS) and antibiotic solution were from Gibco (USA), DMSO (Dimethyl sulfoxide) and MTT (3-4,5 dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide) (5 mg/ml) were from Sigma, (USA), 1X PBS was from Himedia, (India)[11]. 96 well tissue culture plate and wash beaker were from Tarson (India). DMEM medium, Penicillin/Streptomycin antibiotic solution, Trypsin-EDTA was purchased from Gibco (USA), ETBr and Acridine orange was purchased from Sigma Aldrich (USA).

Preparation of polyherbal formulation

The fresh leaves were collected and cleaned with distilled water and shade dried for 1 week. The dried leaves were grounded mixer grinder. The polyherbal formulation was made from different ratios of herbal powders listed in

Table 1. MEDICINAL PLANTS

S.No	Name of the medicinal Plants	Weight
1	*Ficus pumila*	5 gm
2	*Andrographis paniculata*	10 gm
3	*Morindacitrifolia*	7 gm
4	*Lantana camera*	3 gm

RATIO OF POLYHERBAL FORMULATION

Phytochemical Extraction (soxhlet apparatus)

The plant powdered sample (10 g) is used for extraction by Soxhlet apparatus at a boiling temperature. The crude powders were defated with 1 litre of petroleum ether (60°- 80°c) using soxhlet apparatus[12-13]. After defating, the extraction was carried out using 1000 mL of 100% ethyl alcohol (75.0°c) for 4 h. After extraction, the samples were evaporated to remain with important ingredients.

Detection of Glycosides

To 0.5ml of extract, 0.5ml of Benedict reagent is added ad boiled for 2 min. Colour changes and ppt is formed. It indicates the presence of carbohydrate.

To prepare Hydrosalyte

To 50mg of extract, 2ml of conc. HCL is added and kept in water bath for 1 hour and then filtration methods. The filtrate is hydrated.

Born- Trageru's Test

Take 2ml of hydrolysate, add 3ml of chloroform, shake vigorously, then the chloroform layer gets separated. To formation 10% ammonia solution of pink colour indicates the presence of glycosides.

Saponification test

To 1 or 2ml of normal sodium hydroxide, 2mlof extract is added and boiled for 2 minutes. Formation of soap or fat indicates the positive test for saponification.

Detection of Proteins by BradFord Method:
To 500 and 1 of extract, add 5mlof brad ford reagent, Take OD at 575nm.
Detection of Phenol by Biuret Test
To 2ml of extract, 1 drop of 2% cuso4 solution. Add 1 ml of 95% ethanol, then add 2 to 3 sodium hydroxide pellets. Formation of pink colour indicates the test is positive.
SaponTest
To 50 mg of extract,20 ml of distilled water. Shake vigorously for 15 min, at 2 cm layer of foam formation indicates the presence of saponins.
Gum Test
To 100 mg extract. Dissolved in 2 ml of distilled water.2ml of absolute alcohol with constant stirring. White colour cloudy ppt indicates gums & mucilage's.
Cell culture
MOLT 4 (Human blood cancer cells)cell line was purchased from NCCS, Pune and cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), 100 ug/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37^{o}C.
MTT ASSAY
The FALM sample was tested for *in vitro* cytotoxicity, using MOLT 4 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[14]. Briefly, the cultured A549 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (200 µL) into 96-well tissue culture plate in DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the MOLT 4sample in a serum free DMEM medium[15-16]. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After the incubation period, MTT (20 µL of 5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) were aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC50 value was calculated using GraphPad Prism 6.0 software (USA).
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 8 sample in a serum free DMEM medium[17]. The plate was incubated at 37 o C 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.

RESULTS
Polyherbal formulation
The polyherbal formulation was made from seven different types of medicinal plants.Soxhlet extraction was done with various ratios of plant extracts such as, *Ficus pumila (5gm*), *Andrographics paniculata (10gm*), *Lantana camara(3gm*), *Morinda citrifolia* (*7gm)*), After evaporation, totally 4 gm of polyherbal formulation was obtained.
Phytochemical analysis
The extracts of poly herbal formulation were used for the qualitative phytochemical characterization for the identification of the various classes of active chemical constituents, using standard prescribed methods. The positive tests were noted as weak (+), moderate (++), strong (+++) and absent (-).The phytochemical characterization results showed the presence of various phytochemicals such as resins, carboxylic acid, steroids, flavonoids, carbohydrates, protein, saponin and glycosides as shown in

Table 2 PHYTOCHEMICAL CHARACTERIZATION OF POLYHERBAL FORMULATION

S.No	Name of the test sample	Phytochemical analysis	Result
1	FALM	Resin	+++
2		Carboxylic acid	+++
3		Steroids	++
4		Flavanoids	+++
5		Carbohydrates	++
6		Protein	+
7		Biuret	-
8		Saponin	+
9		Glycoside	+

S. No	Tested sample concentration (µg/ml)	OD Value at 570 nm (in triplicates)		
1.	Control	0.444	0.443	0.346
2.	100 µg/ml	0.119	0.119	0.112
3.	90 µg/ml	0.132	0.135	0.121
4.	80 µg/ml	0.145	0.144	0.152
5.	70 µg/ml	0.173	0.176	0.190
6.	60 µg/ml	0.180	0.277	0.256
7.	50 µg/ml	0.228	0.300	0.254
8.	40 µg/ml	0.252	0.298	0.270
9.	30 µg/ml	0.200	0.300	0.225
10	20 µg/ml	0.288	0.286	0.306
11	10 µg/ml	0.444	0.214	0.252

Table 3 ANTICANCER ACTIVITY OF POLYHERBAL FORMULATION

S.NO	Tested sample concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
1.	Control	100	100	100	100
2.	100 µg/ml	28.95	28.95	27.25	28.38
3.	90 µg/ml	32.11	32.84	29.44	31.46
4.	80 µg/ml	35.27	35.03	36.98	35.76
5.	70 µg/ml	42.09	42.82	46.22	43.71
6.	60 µg/ml	43.79	67.39	32.28	57.82
7.	50 µg/ml	55.47	72.99	61.80	63.42
8.	40 µg/ml	61.31	72.50	65.69	66.50
9.	30 µg/ml	48.66	72.99	62.04	61.23
10.	20 µg/ml	70.07	69.58	72.45	71.45
11.	10 µg/ml	108.02	52.06	61.31	73.79

The anticancer activity of polyherbal formulation was carried out against MOLT- 4 at different concentrations to determine the IC_{50} (50% growth inhibition) by MTT assay. Results of different concentrations of polyherbal formulation were shown in Fig. The formation of formazan crystals decreases when the concentration of polyherbal formulation increases in MOLT- 4 cells. MTT assay of polyherbal formulation showed significant effect on MOLT- 4 cell line in a concentration range between 100 µg/ml to 10 µg/ml compared with control. Similarly, polyherbal formulation inhibited the cancer cell growth more than 50%. This polyherbal formulation exerts high cytotoxicity in 100 µg/ml concentration against MOLT- 4 cell line. The IC50 values of polyherbal formulation on MOLT- 4 cell line were 61.91 µg/ml. Taken together, these results showed that the polyherbal formulation are toxic to MOLT- 4 cells and effective in inhibition of cancer cell proliferation[18-19].

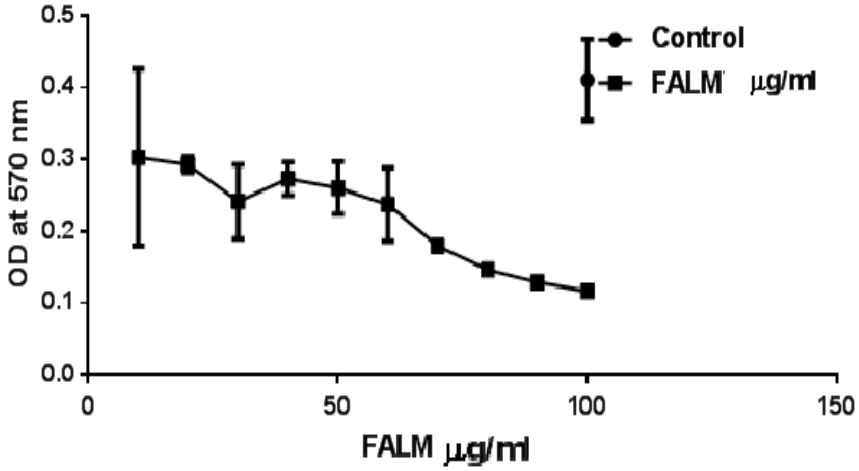


Fig.1 Control Mean OD value: 0.411B. Cell Viability (%)

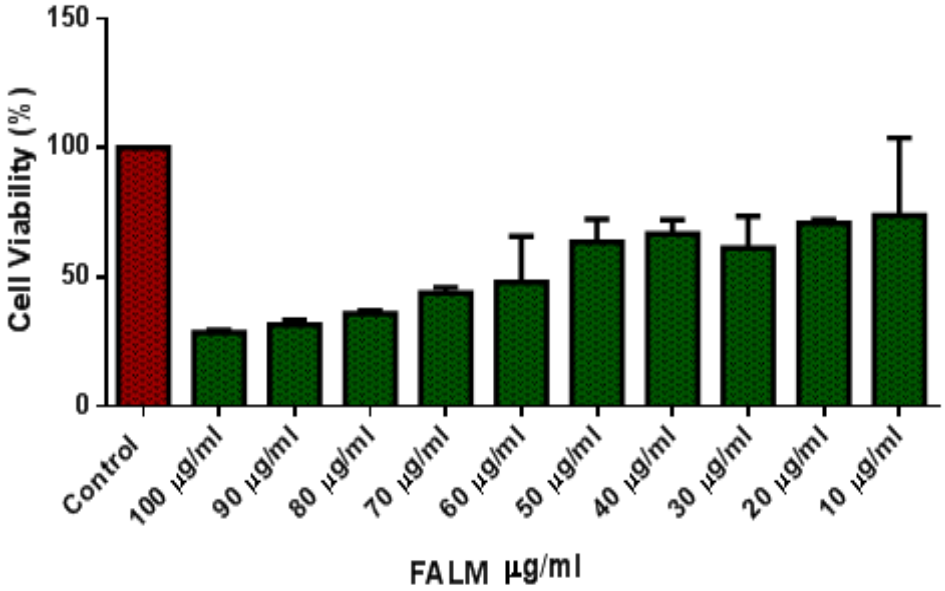
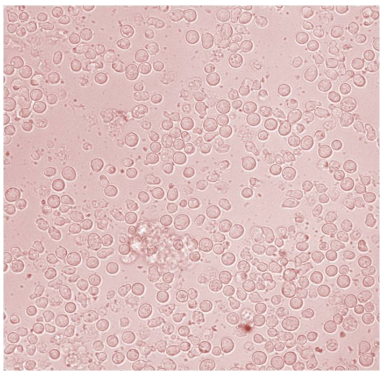


Fig.2 IC50 Value of tested sample: 61.91 µg/ml



Control

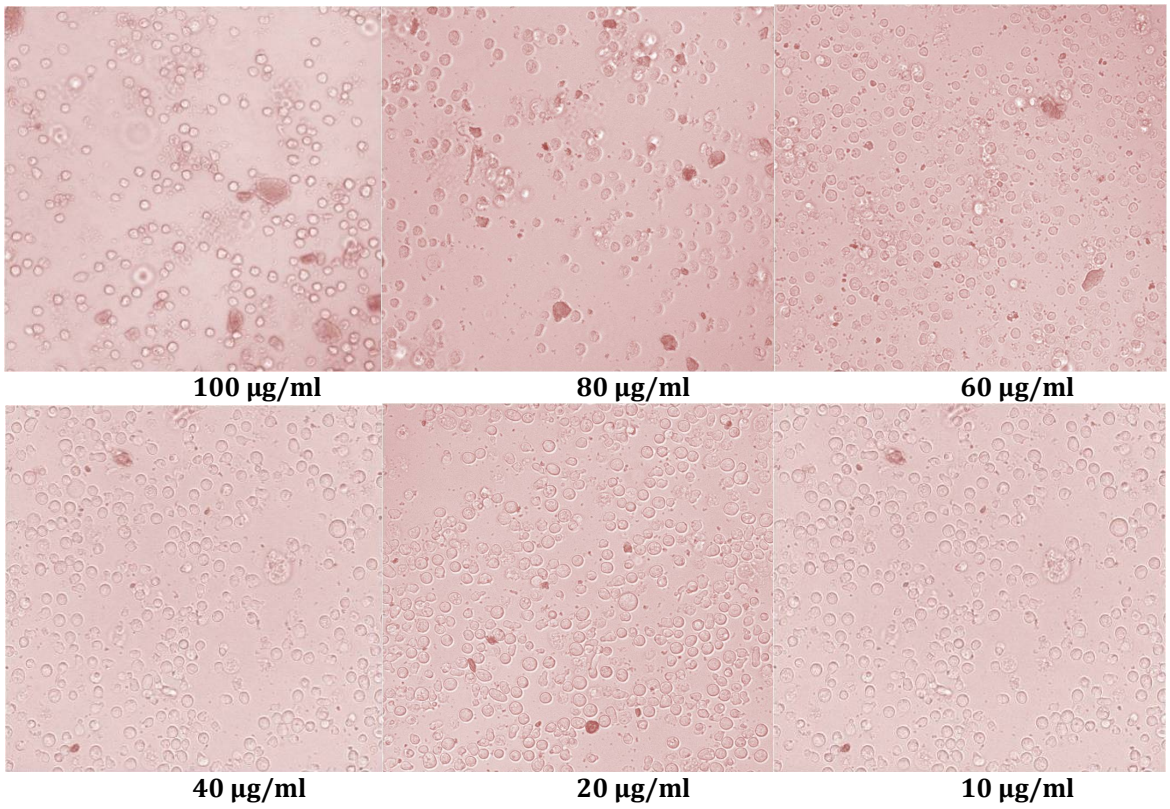
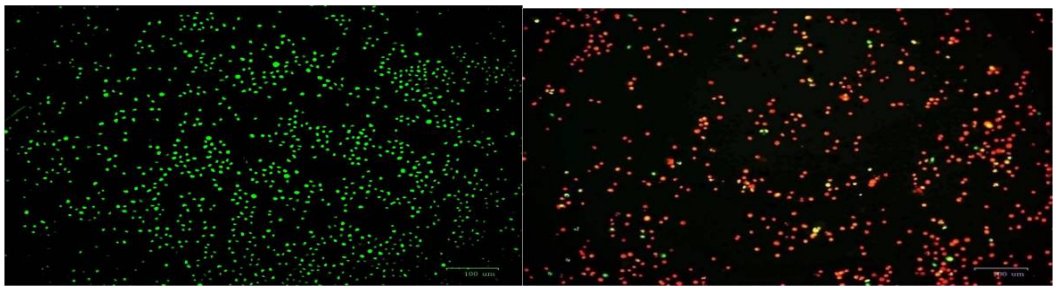


Fig.3 Images of control cells anf polyherbal formulation treated cells

DISCUSSION

The polyherbal formulation treated MOLT- 4 cells were subjected to AO/EB staining. AO will enter the nucleus and stain live cells as green color fluorescence and EB will penetrate the nucleus of dead cells due to loss of membrane integrity and stain as red colour fluorescence. In the present study, the MOLT- 4 cells were treated with 61.91 µg/ml of polyherbal formulation and the cells were examined using fluorescent microscopy. Normal viable cells appeared as green fluorescence with highly organized nuclei[20]. Early apoptotic cells (9.47 %) were appeared as a crescent-shaped or granular yellow-green with AO nuclear staining. Late apoptotic cells (14.21%) were appeared as a concentrated and asymmetrically localized orange nuclear ETBr staining. Necrotic cells (45.96%), Live cells (7.07%) and Dead cells (26.31%) showed uneven, orange-red fluorescence at their periphery without chromatin fragmentation. The IC_{50} values of plant extracts 61.91 µg/ml treated cells showed typical apoptotic and necrotic morphological features such as condensed nuclei, membrane blebbing and formation of apoptotic bodies, which were clearly observed under the fluorescence microscope.



Control Apoptotic and necrotic indexes of 61.91 µg/mlofherbal formulation treated cells
Fig 6. ETBr/Ao staining showing the apoptotic and necrotic indexes of Herbal formulation in MOLT- 4 cells

Table 5. Apoptotic and necrotic indexes of polyherbal formulation treated cells.

S.NO	Live cells	Dead cells	Necrotic cells	Pre apoptotic	Apoptotic
1.	19	50	123	25	43
2.	21	100	139	29	38

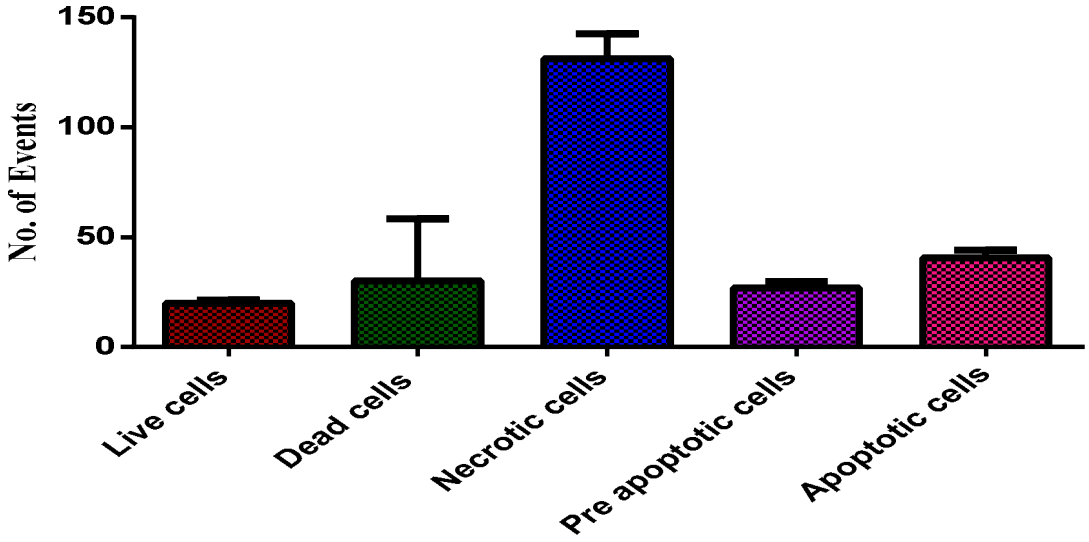


Fig.7. Showing the apoptotic necrotic indexes of 61.91 µg/ml of polyherhabl formulation treated cells

CONCLUSION

The polyherbal formulation was used against cancer in Indian traditional systems of medicines like Ayurvedic, Siddha and Unani. The formulated herbal drugs have become a boon for mankind since ancient times and still are, used worldwide for the treatments of various human ailments. There 7 different types of medicinal herbs such as *Lantana camera, Ficus pumila, Andrographics paniculata, Morinda citrifolia,*was found to be rich in anticancer activity against blood cancer cells. Our polyherbal formulation FALM showed significant anti-cancer activity against the blood cancer cells lines in invitro condition. In order to evaluate the anticancer properties, further research will be continued by following this polyherbal formulation in *invivo* studies using mice and rat.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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