

International Journal of Scientific Research and Reviews

Lupenone Modulates Classic Hallmarks of Cancer in Ascites and Solid Tumor Growth by Restoring Programmed Cell Death and Halting Neovascularization Progression

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ABSTRACT:

Ubiquitously distributed plant phytochemical Triterpenoids are evidential support as beneficial value against several types of human diseases, including various cancers. Anticancer properties of triterpenoids and their anti-inflammatory, anti-proliferative and pro-apoptotic effects have been known both in *in-vitro* and *in-vivo* models. Here we are reporting for the first time role another triterpenoid Lupenone for anticancer activity particularly activating apoptosis and inhibition of new vessel formation which halts the growth of cancer cell. The Lupenone was initially subjected to cell based screening for antiproliferative studies against multiple cancer cells lines of different origin including Lung, Breast, Skin, liver, lymphoma and normal cell lines. Lupenone turned out to induce significant cytotoxicity against lymphoma and melanoma cells. Further, promising antitumor activity of Lupeonone against ascites lymphoma and solid lymphoma tumour model system *in-vivo* resulted the extended survivality of mice in both the model system. The underlying mechanism of tumour inhibition in both the models is due to angiosuppression and activation of cell death as supported by membrane blebbing and condensation of nucleus as well as reduced micro vessel density suggesting the possible application of Lupenone as a potent antitumour agent which could be developed as natural drug for cancer therapy.

KEY WORDS: Lupenone, Cancer, Ascites tumor, Solid tumor, Apoptosis and angiogenesis.

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INTRODUCTION:

Cancer is the reason for the second largest carnage all around the globe and became one of the chief community haleness¹. Preclinical studies have indicated that cancer progression is a multi factorial and multistage event which involves of three distinct stages: initiation, promotion and progression²⁻³. Confrontation to apoptosis and succeeding angiogenesis are considered as significant hallmark of cancerous cell⁴. Irregularity in the cellular homeostasis and cell turnover provokes the cancerous cells to escape from one of the important cellular event termed as apoptosis, constituting un-coordinating cellular metabolism cell escalates angiogenesis process, another physiological event where new blood vessels are sprouts from preexisting one, for their survival⁵⁻⁷. The current treatment procedure comprises radiation, chemotherapy, immunosuppression and surgery. Chemicals used in chemotherapy predominantly exhibit significant sideeffects. Thus, there is a urgent need for substitute agents primarily derived from natural sources to improve the chemotherapy⁸.

Attention towards medicinal dietary herbs and their derivative phyto-chemicals are being increasing and recognized as advantageous for treatment of cancer. Among various edible medicinal herbs, triterpenoids group of phytochemicals are rich reservoir many pharmacological potencies⁹. In route of searching these classes of compounds for anticancer activity, we have found Lupenone as new role in preventing cancer in particularly targeting hallmarks of cancer. Lupenone one such terpenoid known for its inhibitory role in inflammation and used as anti-inflammatory agent for wide range of inflammatory disorder such as Rheumatoid arthritis¹⁰⁻¹². Taking this into consideration and cancer being the inflammatory disorder efforts have been made here to investigate the role of Lupeone in prevention of tumor formation in both ascites and Solid tumor system and cause of tumor inhibition by lupenone. The results inferred that Lupenone targets multiple hall marks of cancer such as resistance to apoptosis and induction of angiogenesis. Such plant based drug will be new hope in development of new drug for cancer treatment.

MATERIALS:

The human Lung Adenocarcinoma cell lines A549, Human breast cancer cell line MCF-7, Human melanoma cancer cells A375, human liver cancer cells HUH-7, and Mouse fibroblasts NIH3T3, Mouse melanoma B16-F10 and Mouse macrophages Raw264.7 cell were procured from National Center for Cell Science (NCCS), Pune, India. The MTT, (3-(4,5-dimethylthiazole-2-yl)-2,5-

diphenyltetrazolium bromide), NaHCO_3 , Sigma Aldrich, USA. DMEM (Dulbecco's Modified Eagle Media), FBS (Fetal Bovine Serum), Antibiotic-antimicotic solution, Trypsin-EDTA solution, from Invitrogen (Gibco), USA. Anti CD31 from Santa cruz, USA. Immunostaining kit from Leica Biosystems, Germany. All other chemicals used in the current study is of analytical grade. All the experiments were performed at minimum three independent times and analyzed. All bright field and fluorescence image were taken EVOS FL cell imaging, Thermo Scientific, USA and results were assessed by ImageJ software.

METHODS:

Cell culture and antiproliferative studies

The cell lines namely DLA, A549, B16F10, A375, HUH-7, MCF-7, RAW 264.7 and NIH 3T3 were cultured in DMEM media providing 10% FBS including NaHCO_3 (0.37 %) , antibiotic and antimicotic agents, maintained with supply of 5 % of CO_2 . The cells were treated with increasing concentrations of Lupenone (5-100 μM) along with suitable vehicle and positive control 5-fluorouracil and analysed for cell proliferation and cytotoxicity by MTT assay. Cells were treated with or without compounds and incubated for 48 h. MTT reagent (5mg/mL) was added and the color change due to proliferating cells was estimated. Experiments were repeated for three times and statistically analyzed by standard methods¹³.

Experimental Animal and ethics

Swiss albino male mice weighing about 25 ± 2 were housed with standard laboratory conditions and used for the experimentation. For all animal experiment procedures was approved by the Institutional Animal Ethics Committee, National College of Pharmacy, Shimoga, India, in accordance with the CPCSEA guidelines for laboratory animal facility (NCP/IAEC/CL/101/05/2013-14).

Lethal dose studies of Lupeonone

Triterpenoid Lupeonone was subjected to short term acute toxicity studies in normal swiss albino mice which divided into 5 groups (n=6) by injecting intraperitoneally (ip) and LD_{50} was assessed as instructed by standard CPCSEA guidelines. The side effect of Lupeonone on normal swiss albino mice injected with the compound (100 mg/kg body weight, i.p) for 10 days were monitored. The

physiological impact of treatment on mice liver and spleen parameters were assessed by histopathology. Obtained values were represented as mean \pm SEM.

Animal models for cancer and mode of treatment

The efficacy of anticancer potentiality of the Lupenone was verified against DLA ascites tumour and solid lymphoma cells *in-vivo*. The DLA cells were maintained separately in the peritoneum of mice by injecting 200 μ l ascites. Murine DLA cells grown *in-vivo* were administered (40 and 60 mg/kg body weight i.p) 3 doses on alternate day after tumor onset as reported earlier¹⁴.

Solid tumour induction was developed as reported earlier¹⁴. In brief, ascitic DL cells from donor mice and injected into the thigh of the animals subcutaneously to develop solid tumour. The experimental animals grouped separately and administered with the Lupeonone (40 mg/kg and 60 mg/kg body weight i.p for 10 doses). After tumor onset the mice, tumour volume was measured on every alternative day. Finally at the end of 50th day after tumour development animals were sacrificed and tissue along with liver and spleen were collected and analyzed.

Peritoneal angiogenesis assay and IHC

The reduction in neovessel formation in peritoneum of mice bearing DLA treated with or without Lupeonone were documented and further formalin fixed tissue was processed for H&E as well as CD31 immuno staining for MVD measurement¹⁵. The MVD were measured by blind folding method and documented.

Nuclear Staining by Acridine Orange and Giemsa.

Differences in control and Lupeonone treated Nucleus of ascitic cells *in-vivo* was measured by Acridine orange and Geimsa stain^{14 and 16}. In brief, harvested DLA cells either treated or untreated with Lupenone (40 and 60 mg/Kg b.wt *in-vivo*) smeared, fixed with methanol and acetic acid (3:1) and stained with either acridine orange or giemsa and images were taken in EVOS fluorescent microscope.

Statistical analysis

Values were expressed as mean \pm standard deviation (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by 2-tailed 13 Student's t-test. MS excel 8.1 version software was used for data analysis and Statistical significant values were expressed as *p < 0.05 and **p < 0.01.

RESULTS AND DISCUSSION.

Lupeonone exhibits antiproliferative activity against Lymphoma and melanoma cells.

An ideal anticancer agent should be capable to inhibit, delay or reverse the cancer progression through its proper cytotoxicity without causing any side effects and with specific target like apoptosis-induction and angio-prevention properties¹⁷. In unearthing the novel class of anticancer drugs, especially cytotoxic molecules, significant differences in normal and cancer cell behavior is considered for the drug development process. Identification such cytotoxic molecules led the development of many anticancer therapeutics potentials from several decades. During the last few decade growing number of biologically active molecules are found to have cytotoxicity against a variety of tumor cells⁹. These are highly multifunctional and the antitumor activity of these compounds is measured by their ability to block nuclear various hall marks of cancer like activation, induce apoptosis, inhibit signal transducer, and activate transcription and angiogenesis is thoroughly investigated^{4 and 9}. In the current study another class of triterpenoid Lupenone has been investigated for cancer prevention.

Since cancer being a multiple syndrome and effects many organs and etiopathology of the disease is varies from one type of cancer to other type of cancer⁴. This could be due to originality, modified genes and several such factors. Being a complex in pathological condition, its obvious that mode of treatment should also be different for each type. Ultimate goal of this is to develop a cancer specific drug particularly masking the cause of molecular event. Hence forth large number of drugs is in the market for each cancer¹⁸. Taking this into consideration here we have approached cell based screening against multiple cancer cell lines such as Lung, Lymphoma, Melanoma , liver and Breast. Serdepindity is its effective against Lymphoma and Melanoma and not against any other cancer cell as well as incentive to normal cell which is ideal for drug development process Screening against various cancer cell lines which includes A549, B16F10, HuH7, A375, MCF7 DLA cells as well as normal cell lines such as NIH3T3 and Raw 267 cell. Lupeonone exhibited significant antiproliferative and cytotoxicity efficiency against DLA and B16F10 and A375 cell lines with average IC_{50} of 9 μ MMTT assay but no significant results were noticed in HUH7 and MCF7 cells specifying the action of Lupenone on melanoma and lymphoma cells and not with any other cells. Further action of the lupenone against normal cells exhibited no sensitivity of lupenone which is important for drug development process Fig:1. Thus, our studies using two cell lines of different origin suggest that

irrespective of the cancer type, Lupeonone could induce cytotoxicity, as shown by three independent assaying methods and further investigated for antitumour effect.

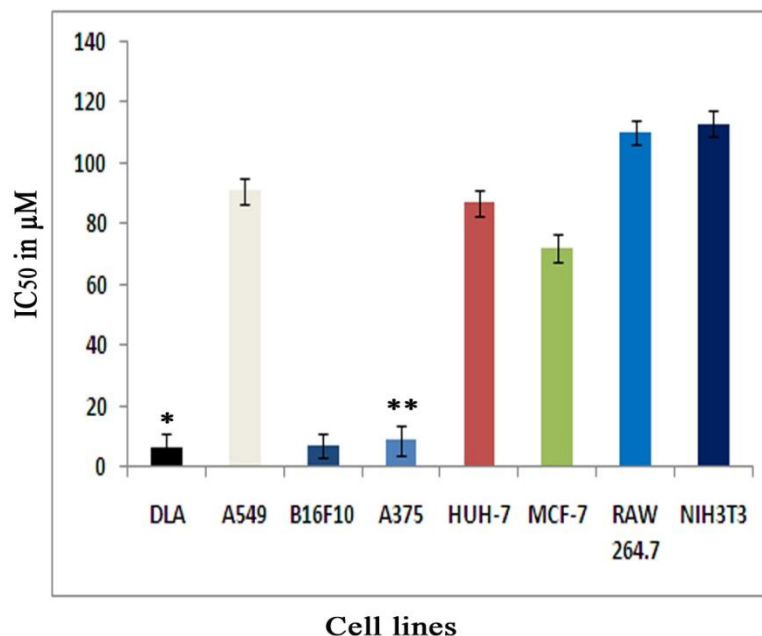


Fig 1: Lupeonone exhibits the antiproliferative effect against DLA and B16F10 and A375 cells:

Lupenone relapse the ascites and solid tumour in vivo

Ascites and solid tumour models of mice are best valuable system for initial screening and have a critical role in drug development. This rationale and hierarchical approach beginning with toxicology as well as pharmacology investigation is necessary for identification of therapeutic targets as an outcome of clinically relevant parameters¹⁴. Here we have chosen mouse ascities and solid lymphoma since the lupeonone was able to have an effect against lymphoma as evident by antiproliferative screening. These model system establishes local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration and a progressive ascites fluid formation after tumour implantation¹⁹ which serves as nutritional requirement of growing tumour cells and thereby establishing the cancer progression. Lethal dose of Lupeonone was fixed and it was assured that no toxicity induction by the treatment by testing against normal cells. Lupeonone regressed the tumour growth was noticeable at low dose 40mg/kg.b.w and 60mg/kg.b.w by decreasing the ascites secretion and reducing the cell count with increased survival of animals in dose dependent manner Fig 2.

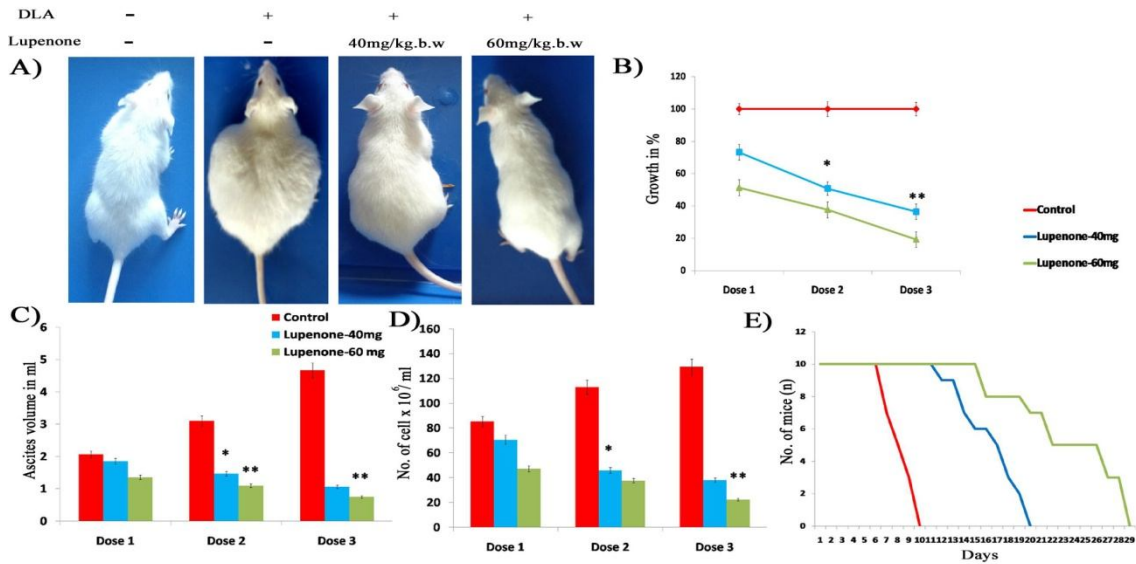


Fig 2: Lupeonone inhibits ascites carcinoma cells: A. Morphology of tumor mice B. Reduced tumor growth C. Decreased ascites secretion D. Number of tumor cells. E. Kaplan–Meier graph showing the survivability of mice.

The solid tumour a suitable reliable representation of histological examination for cancer and thereby providing the swift action of drug delivery¹⁴. The concern in drug development process is target specific in action minimizing the side effects. Therefore the antitumour potency of lupeonone was evaluated in solid DL tumour model system. The capacity of Lupeonone was reassessed in developed solid tumour treated with lupeonone for 6 doses every alternative days at 40mg/kg.b.w and 60mg/kg.b.w. Detectable reduction of animal thigh tumour morphology seen in the lower doses treatment, most diminished growth of tumour observed in 60mg/kg.b.w. The mass of the thigh tumour of animals evidenced the potent activity of Lupeonone against solid tumour (Fig 3-B&C) and correspondingly enhanced the increasing survival days of the animals Fig: 3.

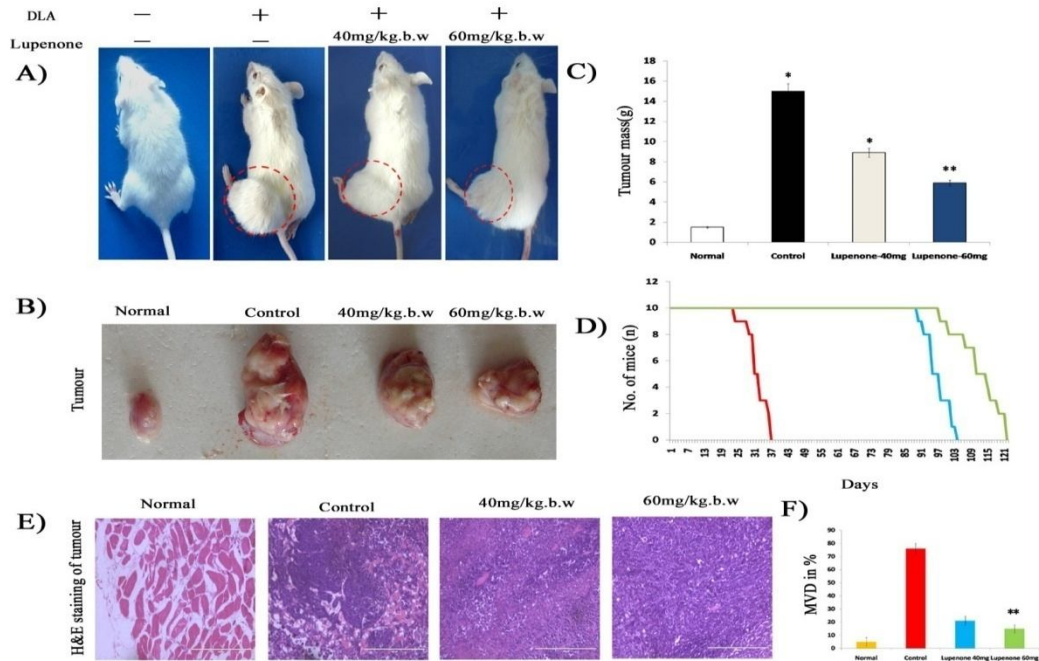


Fig 3:Lupenone efficiency on retrogression of solid tumour: A. Tumor morphology of animals B. Dissected thigh tumour C. Tumour mass D. Kaplan–Mayer graph for survivability E &F. H&E staining for MVD.

The cytotoxicity parameter was after *in-vivo* treatment in liver and tissue was totally negligible Fig: 4.

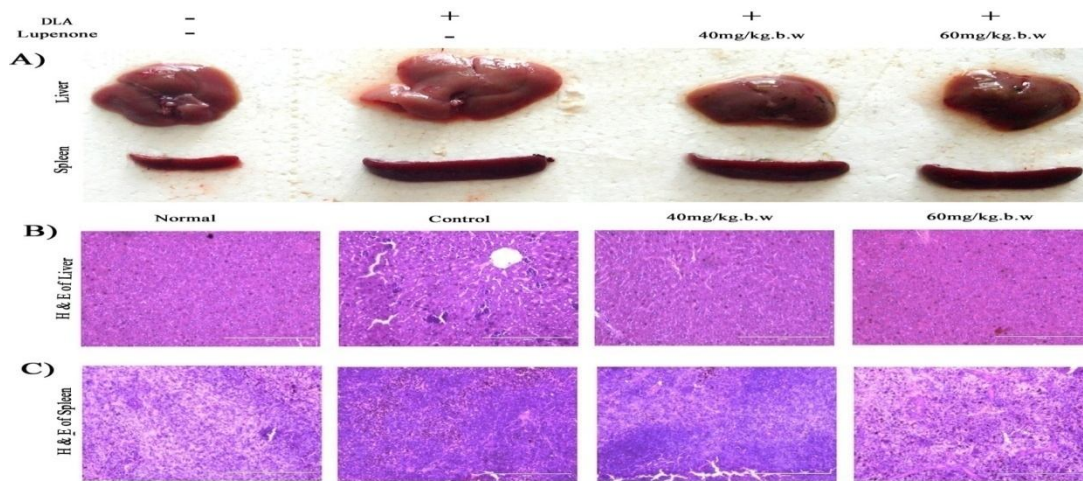


Fig 4: Non-toxicity of lupenone to the organs of the mice:A. Photographs of liver and spleen B. H&E staining of liver and (C) spleen.

Lupenone targets multiple hallmarks of cancer.

Establishment of tumor and metastasis are dependent on progressive neovasucarization and increased neovasculature will allow not only increase in tumour growth but also enhances haematogenoustumourrembolization. Thus blocking tumour vasculature will arrest the tumour establishment and thereby eventual events of cancer. Measurement of MVD is well established bio marker for pathological examinations in tumour models assess the prognosis of the disease^{4 and7}. In this investigation Lupeonone has potent antitumour efficacy and activation of antiangiogenesis could be one of the possible underlying mechanism of tumour inhibition. Since the angiogenesis is evident in the inner lining of the peritoneum of the DLA tumour bearing mice and it is a reliable model to study the angiogenesis dependent tumour growth²⁰, we validated the effect of lupenone for angio-prevention activity. Tumor induced Peritoneal angiogenesis was also drastically reduced in Lupenone treated animals. The histological study and IHC staining with CD31, and endothelial specific marker was clearly indicated the dose dependent decrease of the microvessel density compared to untreated tissues Fig 5.

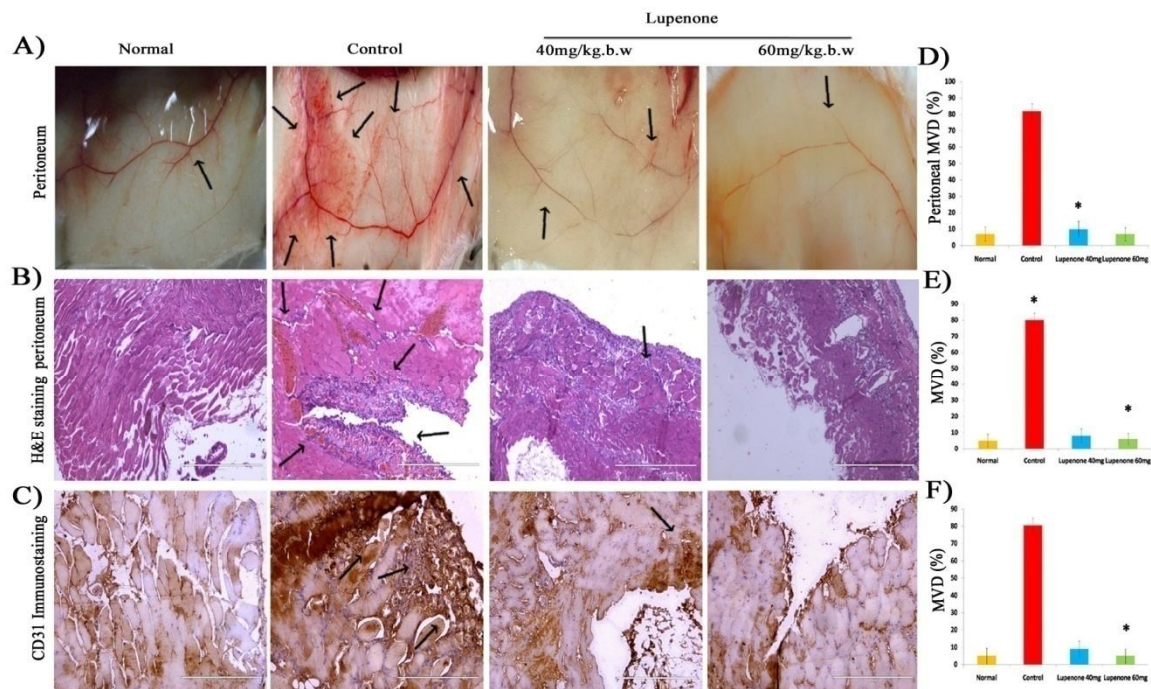


Fig 5: Anti-angiogenic effect of lupenone in ascites tumour: A. Decline MVD in peritoneum B H&E staining of peritoneum C) Immunostain of with anti- CD31 antibody with D, E & F. respective measurement.

The lost apoptosis and angiogenesis are the two important characteristics which promotes the establishment of the tumour and there is a direct correlation between these two characteristics⁴. Thus inhibition of angiogenesis may leads to promotion of apoptosis resulting in cell death there by tumour inhibition. Several anticancer drugs *with* these dual effect used in current cancer therapy and many are in clinical trials⁹. When we verified the possible proapoptotic effect of lupenone on DLA cells by Acridine orange and Giemsa stain we have found condensed nucleus and membrane disruption changes which are typical hall marks of apoptosis Fig6.

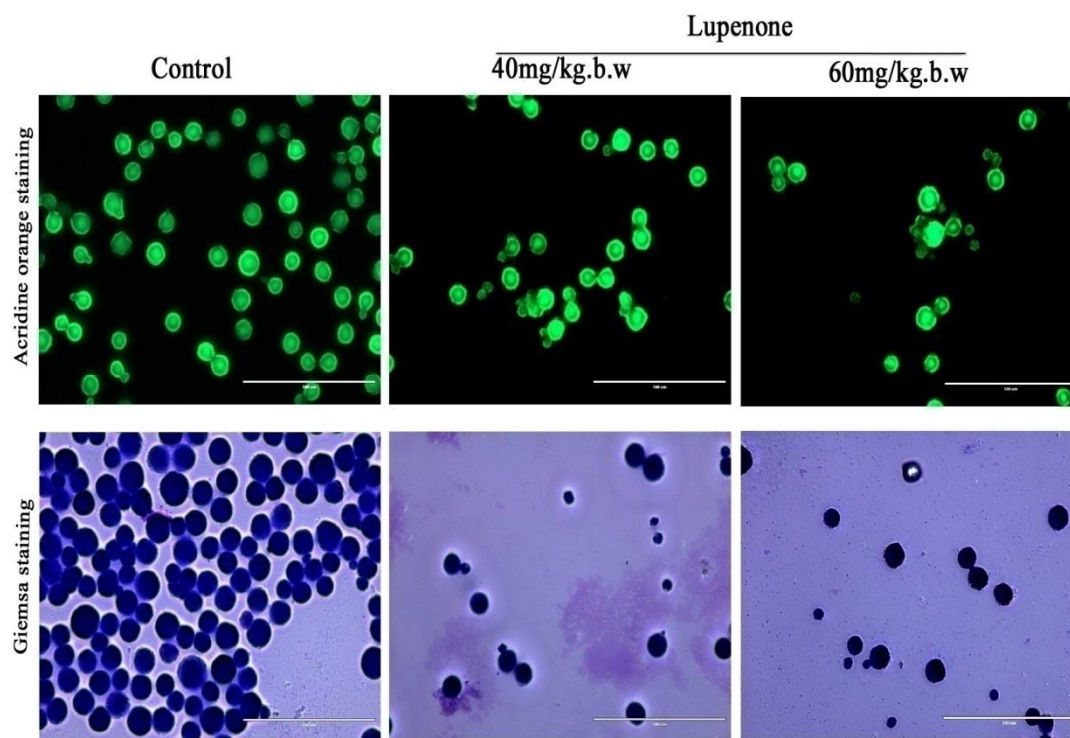


Fig 6: Diminishing the ascites tumour cells through apoptosis.A. Acridine orange and .B) Giemsa stain

CONCLUSION:

Triterpenoids are known pharmacologically active substances and novel class of triterpenoid Lupenone exhibited its new role particularly inhibiting Lymphoma and Melanoma types of cancer *in-vitro* and Lymphoma *in-vivo* both ascites and solid tumor. The mechanism of tumor was identified as a promising anti cancer molecule with multiple mode of actions such as antiproliferative, angiopreventive effect together with its remarkable apoptosis inducing action, making it as great interest for further studies.

CONFLICT OF INTEREST:

Authors exhibits no conflict of interest

ACKNOWLEDGMENT:

Dr.B.T. Prabhakar thankful for the research support given by UGC-MRP (F.No.41-507/2012), Rakesh Hanumaiah acknowledges the Rajiv Gandhi National fellowship(RGNF)(No. F1-17.1/2016-17/RGNF-2015-17-SC-KAR-19249 dated: 12/01/2016) for the financial aid and support.

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