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Cytotoxicity and Mitochondrial-Mediated Apoptosis Induced by Ethanolic Leaf Extract of *Barleria lupulina* Lindl. in Human Leukemia Cells Via Reactive Oxygen Species Generation

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Abstract

Background: *Barleria lupulina* Lindl. (Hop-headed) is a small shrub, possess potent anti-inflammatory, analgesic, anti-leukemic, antitumor, anti-hyperglycemic, anti-amoebic, virucidal, diuretic, bactericidal and antibiotic properties.

Methods: Cytotoxicity, bioactive assay and genetic analysis of *B. lupulina* were investigated in the present communication. The leaf extract was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Neutral red uptake (NRU), DNA fragment, reactive oxygen species (ROS) generation, mitochondrial membrane potential (MMP) assay, gene expression analysis and cDNA synthesis to evaluate anti-cancerous potency using cancerous THP-1 cell lines in vitro and in vivo.

Results: HPTLC analysis reveals four spots and GC-MS analysis displayed the presence of eleven bioactive compounds among which benzofuranon, hexadecanoic acid, ethyl 9,12,15-octadecatrienoate, and 3,7,11,15-tetramethyl-2-hexadecanoic acid were the most prominent compounds. The ethanolic extract showed significant cytotoxicity ($P<0.5$) against THP-1 cell line at a concentration of 1mg/mL. The cells were also observed for apoptosis through DNA fragmentation in *B. lupulina* treated cells.

Conclusions: It can be concluded that if the dose range was further refined within the range of 100-1000 µg/mL there could be dose at which the entire population of the THP-1 cell line would be apoptosis induced. The extract induced ROS in the cells after 30 minutes of exposure displaying cytotoxic effects and DNA fragmentation assay.

Keywords: *Barleria lupulina*, cytotoxicity activity, MTT, NRU, ROS, MMP, THP-1 cell line.

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Background

Oncogenes stimulated the uncontrolled growth of cells resulting in tumor that is the causing cancer leading the death of the sufferers. About one-half of all men and one-third of all women in the world develop cancer. Now a days, millions of people are living with cancer or have cancer. It is quite dangerous for all people and an easy task to treat the ailment. Using herbals for the treatment of malignancies is common in many cultures especially in India, because some herbal products contain abundant anti-cancerous compound. In addition, useful compounds from these herbals are being used in production of various modern drugs. Herbal medicines constitute a major substitute for cancer prevention and treatment around the globe. The effect of plant extracts as anti-cancerous was widely studied owing to their low toxicity and side effects [1]. Hence, such studies investigating medicinal herbs have been steadily held with interests.

Barleria lupulina Lindl. (Family: Acanthaceae) is an important medicinal plant distributed in the mountains of southern, western and central India. The principle constituents of leaf and stem circumscribe the presence of glycosides i.e. barlerin, shanzhiside, methyl ester, etc**.** In folk medicine, *B. lupulina* has been used traditionally as an anti-inflammatory [2], antidiabetic, analgesic, antimicrobial and anti-ulcerogenic agent [3]. Some constituents of *B. lupulina* have been tested for antitumour activity in different carcinogenic models. *B. lupulina* has also been reported to possess a potent Antimicrobial [4, 5, 6], Anti-inflammatory [2, 7], Analgesic, Antiulcerogenic [7], Antidiabetic [8], Neuropharmacological [9], Toothache [10], Antibacterial [11], Anticancer [12], Anti-artheritis, Acute and sub-chronic diuretic [13], Anti-viral [14]. However, very little is known regarding the molecular mechanisms by which they may exert their

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antitumourigenic effects. The leaf extracts have been reported to bear anti-cancerous properties on Hep G2 cell [15]. Present work was aimed to evaluate anti-cancerous potency of *B. lupulina* leaves using cancerous THP-1 cell lines in vitro and in vivo.

Methods

Plant material, extraction and Apparatus

B. lupulina leaves collected from the Botanical Garden, Department of Botany and Microbiology, Gurukula Kangri Vishwavidyalaya, Haridwar (India) (specimen identification No. Bot. & Micro/199/2016) and washed with running tap water to remove the adhered dirt, dust and other foreign material. Plant materials were dried in shade at room temperature and homogenized to get fine powder. The powdered material was subjected to hot extraction in Soxhlet continuous extraction apparatus with ethanol solvents for 48 - 72 h. The extract was filtered and evaporated under vacuum distillation unit at 60°C.

The preliminary phytochemical, High Pressure Thin Layer Chromatography (HPTLC), Gas Chromatography - Mass Spectroscopy (GC-MS), dose preparation for cell line and culture condition, MTT assay, Neutral Red Uptake (NRU) of extract was done as per following Kumari and Dubey [12] and trypan blue exclusion dye method as per Jayashree and Thenmozhi [16].

DNA fragment assay

The cells were cultured (1×10^5 cells/mL) in the 6-well microtitre plate. After incubation for 24h, different concentrations of extracts were treated followed by pipetting out the confluent cells in the plate without scratching. Lysing buffer was added once

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about 100 µL for extracting the cells through scratching. Same step was followed again and collected in labeled Eppendroffs. Then 2µL RNAse was added in each Eppendroffs tube and kept for incubation for 1 hr. Then 2µL proteinase K was added and the Eppendroff incubated for 1 hr. Buffered phenol/chloroform/isomyl propanol was added followed by centrifugation at 10,000 rpm for about 10 minutes. The supernatant was isolated to which 50 µL sodium acetate and 200 µL isopropanol were added followed by centrifugation. The pellets were isolated, 70% ethanol was added and the contents were centrifuged. The collected pellets were kept for air drying. Thereafter, Tris-EDTA buffer was added to the dried pellets and was used for quantifying DNA using nanodrop.

The loading samples were created according to amount of DNA in Eppendroffs and dye was added. Gel electrophoresis unit was prepared. The gel was casted and the samples and the ladder (as a control) were loaded into gel at 80-100 volt. Further, the gel was analyzed under gel documentation system.

Reactive oxygen species generation

Cell suspension (100 µL) was seeded in each well of 96 well plate (20,000 cells per well) and was given the treatment No. 4 (100 µg/mL) of *B. lupulina* extract (5µL) with different time intervals (i.e. 0 minute, 30 minutes, 1, 3, 6 and 24 h). Thereafter, medium was removed and 2µL of DCFDA was added and incubated for 30 minutes. Fresh 1×PBS in each well was added after removing the previous one. The florescence was recorded at 485/528 nm with the help of microplate reader (Reactive Oxygen Species (ROS) detection reagents.

Mitochondrial membrane potential assay

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Cell suspension (100 µL) was seeded in each well of 96 well plate (20,000 cells per well) and left in CO_2 incubator for 24 h. After overnight incubation, treatment No. 4 (100 µg/mL) of *B. lupilina* extracts (5 µL). The plates were treated and incubated for 24 h. The medium was removed and 100 µL of 1× PBS was added in each well and 5 µL of Rhodamine 123 (dissolved in 1000 µL of DMSO and 1000 µL of 1 × PBS) was added in the control and treatment wells and incubated for 30 minutes. Thereafter, the plate was read at 500 nm (excitation) and 526 nm (emission) with the help of microplate reader [17].

Gene expression analysis with quantitative PCR

The cells were cultured (1×10^5 cells/mL) in the 6 well plate and was treated with 100µg/mL concentration of *B. lupulina* in each well. The control and the treated cells were pooled from the plate and was treated with Trizol reagent and was stored at -20ºC. After the removal of growth medium from the culture dish, 1ml Trizol reagent was added to the cells. 0.2 ml chloroform was added per 1ml of Trizol reagent used for homogenization. It was incubated for 3 min at room temperature followed by centrifugation at 1200g for 15 minute at 4°C. The aqueous phase of the sample was removed by angling at 45 degree and pipetting the solution out. The aqueous phase was placed into the new tube and the spots for RNA isolation was proceeded including RNA precipitation, RNA wash, and RNA resuspension [18].

cDNA Synthesis

After isolation of total RNA, 1µg RNA was used for synthesis of cDNA with the help of Thermo Scientific Verso cDNA kit. 1µg RNA was first mixed with the primers of interest and was heated at 65°C for 5 mins and then 5X cDNA synthesis buffer, dNTP

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mix, RT enhancer (DNAse enzyme) and verso enzyme mix were added in a PCR tube and final volume was made up to 20µl. First strand synthesis (cDNA synthesis) was carried out by putting the mixture at 42°C for 1 hour. Synthesized cDNA was quantified using nanodrop.

Quantitative PCR setup

Synthesized cDNA (100 mg) was mixed with β-Actin/ c-MYC primers (0.5 µM), SYBR green master mix 2X and final volume was made upto 20 µl. Sample was acquired in Life Technologies Real Time PCR System and results were presented as CT, values calculated from the amplification plot and nonspecific products were observed with the melt curve analysis.

Statistical analysis

Data were expressed as mean standard deviation (SD). One-way analysis of variance ANOVA were performed by using GraphPad Prism5 and statistical significance of results measured by using Duncan's multiple range, significance test ($P < 0.05$).

Results

Preliminary phytochemical test

Ethanolic leaf extracts revealed the presence of alkaloids, steroids, saponins, cardiac glycosides, tannins, aminoacids, sugars and flavonoids.

HPTLC analysis

HPTLC analysis of extracts revealed the presence of alkaloid and terpenoid group of compounds in different spots observed at 366 nm in fluorescence and normal modes (254 nm). These spots showed different phyto-constituents along with their R_f values

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were0.2, 18.6, 18.3, 3.1 (Fig. 1) and their area of percentages were, 14.06, 21.79, 40.80, 23.35.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

On the basis of NIST library various phytoconstituents were identified through GC-MS analysis from ethanolic leaf extract such as tetradecane, 1,H-3a-7-methanoazulene, cis-thiopsine, benzofuranon, hexadecane, 3,7,11,15, tetramethyl-2-hexadecanoic acid, 3-eicosyne, hexadecanoic acid, oxiranehexadecyl (phytol), ethyl 9,12,15 octadecatrienoate and squalene (Fig. 2).

Trypan blue exclusion dye

The effect of ethanalic leaf extract was very effective after the treatment of Trypan blue dye on THP-1 cell line. The highest percentage of viable cells was 90.09 at 0.1 µg/mL and the lowest was 25.13 µg/mL after 24 h treatment while, the number of viable cells were 95.25 in control well at 0 h shown in (Fig. 3 and Fig. 4).

MTT assay and NRU assay

The ethanolic extract of *B. lupulina* induced cytotoxic effects on THP-1 cell line at concentration of 500- 1000 µg/mL and IC_{50} at 820 µg/mL (Fig. 5a). Different types of the cytopathic effect of leaf extracts including cytoplasm vacuolation, cell shrinkage, lysis and death in THP-1 cells (leukemia cell line) were observed. The cytotoxicity of ethanolic leaf extract was analysed by NRU assay on THP-1 leukemia cell line. It revealed the cytotoxic effects of ELE of *B. lupulina* at all the doses from 0.1 µg/mL to 1000 µg/mL effective on the THP-1 leukemia cell line (Fig. 5a and 5b).

DNA Fragmentation assay

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The DNA was isolated from the treated cells and subjected to agarose gel electrophoresis. A ladder formation is the characteristic of apoptosis. The ladder is formed due to the fragmentation of DNA by the cytotoxic effect (THP-1 leukemia cell line) of ethanolic leaf extract (ELE) of *B. lupulina*. The fragmentation was visible at the 100 µg/mL concentration of extract. DNA of THP-1 showed intact form in all the concentrations except in the concentration of 100 µg/mL which showed some amount of contamination in form of RNA or proteins. Fig. 6 shows that DNA band in all the concentration is in the pure form.

Reactive oxygen species (ROS) generation

Exposure of xenobiotic compounds induces a stress signal in the cells and as a defense system cells start producing reactive oxygen species (ROS) which in turn helps the cells to eliminate the negative effect of the xenobiotic compounds. However, in the case of continuous production of cellular ROS, cellular system starts deteriorating and thus inducing apoptosis in the cells. In the present study, *B. lupulina* extract at 100 µg/mL induced ROS in the cells after 60 minutes of exposure and its effect continued upto 180 minutes (Fig. 5c).

Mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was estimated with Rhodamine 123 dye to evaluate the effect of *B. lupulina* extract on THP-1 cell line. Rhodamine 123 absorption indicates the integrity of mitochondrial membrane. Thus, it is a direct measurement of apoptosis. Results indicated that *B. lupulina* extract slightly increased the mitochondrial membrane potential in the THP-1 cells at 100 µg/mL and in dose-dependent manner (Fig. 5d).

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Gene expression analysis

The RNA was isolated from the control and treated cells and cDNA was synthesized, further this cDNA was used in real time PCR or quantitative PCR for further analysis of gene expression viz. β-actin and c-Myc. Amplification plot indicated the suppression of c-Myc gene by 20 fold as compared to control. Melt curve of the samples revealed the presence of few non-specific products that may be due to annealing temperature of the primer.

Discussion

In medicinal plants different types of phytochemicals constituents are found in medicinal plants viz., alkaloids, saponins, tannins, flavonoids, steroids, glycosides, etc. But the chemicals components of plants may differ in different environmental/stress conditions. Extractions of bioactive compounds also depend on the solubility of organic solvents. In primary screening, different phyto-constituents of ethanolic leaf extract of *B. lupulina* were separated on HPTLC plates that were terpenoid and alkaloid groups of compounds. The extract revealed four spots on HPTLC plate and similar bioactive compounds were found by Sur [19]. They also reported the presence of steroid, terpenoid, glycoside, flavonoid, tannin and carbohydrate which were corresponds to six spots from ethanolic extract of *B. lupulina* under UV (254 nm) in preparative TLC.

Medicinal plants contain various phytochemicals i.e. alkaloids, saponins, tannins, flavonoids, steroids, glycosides whose composition can be vary along the environmental/stress conditions.

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Some other bioactive compounds viz., benzene (1-methyl decyle), benzoic acid 4-methoxy-methyl ester, propenoic acid, benzyl benzoate and 2 (4H)-benzofuranone were identified from acetone and methanol-soluble extracts of *B. lupulina* through GC-MS [12]. Besides, slightly different constituents such as, tetradecane, 1,H-3a-7-methanoazulene, cis-thiopsine, benzofuranon, hexadecane, 3,7,11,15, tetramethyl-2-hexadecanoic acid, 3-eicosyne, hexadecanoic acid, oxiranehexadecyl (phytol), ethyl 9,12,15 octadecatrienoate and squalene have been found in this communication. These all bioactive compounds have antimicrobial effects. More or less similar phyto-constituents have been reported by Kumari and Dubey [15].

The percentage of viable cells decreased with the increase in the concentration of extract on the THP-1 cell line. The extract has the ability to caused apoptosis in THP-1 cell line which was observed as cell membrane blebbing under the microscope. The bio-active components of extract may be responsible for the apoptotic elimination of cancer cell.

Choudhury et al. [20] have also reported the highest inhibition at 100 µg/mL on DLA cell line. Ethanolic extract of *B. lupulina* and *Calotropsis gigantean* leaf mixture exhibited the minimum number of DLA-cell viability. In this observation, THP-1 cell viability started decreasing at minimum concentration (0.1 µg/mL) of extract. This is the first report on THP-1 cells line because in above experiments method was same but cell line was different.

NRU assay revealed cytotoxic effects of ethanolic extract of *B. lupulina* even at the lowest dose (i.e. 0.1 µg/mL) and the IC_{50} values was 580 µg/mL. The IC_{50} value of ethanolic extract of 650 µg/mL against Hep G2 cells earlier been reported. The lowest

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IC_{50} value was recorded against THP-1 than Hep G2 cells. Therefore, THP-1 cells were more affected with the treatment of ethanolic extract than the Hep G2 cells [15].

The morphological changes in the cells were observed in treated cells which showed extensive cell death. The cell viability of the cancerous cells exposed to *B. lupilina* extracts decrease in the cells.

The methods, temperature and time of extraction, solvent type, concentration of solvent, etc. affect the extraction of phytochemical constituents. Ethanolic extract of *B. lupulina* exhibited the highest selective index (SI) (781.5) with lowest (IC_{50}) 50% inhibitory concentration dose (0.02 µg/mL) against HSV-2 cells [21]. It was the lowest IC_{50} value. In contrast, IC_{50} value was higher because cell lines and solvent system were different and extraction procedure was also different. However, it has been proved that every cell line contains ability to survive in stress condition as well as exposure of treatment of extracts.

The antiviral activity of *B. lupulina* and *Clinacanthus nutans* extract against five HSV-2 isolates (IC_{50} of 442.2-987.7 µg/mL) was studied by Yoosook et al. [4]. *Clinacanthus nutans* did not show any activity against these virus strains. Jayavasu [22] also observed high IC_{50} of *C. nutans* extract compared to *B. lupulina*. Multifarious ways of cytotoxicity of *B. lupulina* extracts alone or in combination with other plant extracts has also been reported by Maity et al. [23]. Who reported the effect of combined mixtures of ethanolic extracts (EECGL+EEBLL) and water extract (WECGL+WEBLL) of *Calotropis gigantea* latex and *B. lupulina* leaf extract on short-term *in vitro* cytotoxicity on DLA cell line. The extracts at two doses (100, and 150 µg/mL) and 5-flurouracil (0.5µg/mL) showed significantly fewer viable tumour cells than the DLA-control group.

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The combined mixtures of ethanolic extract of *B. lupulina* and *C. gigantea* showed the minimum number of DLA-cell viability. The *Allium cepa* leaf extract has *in vitro* cytotoxic, apoptotic and antiproliferative potential on Dalton's Ascitic lymphoma cell as well as DLA-bearing Swiss albino mice.

A Similar work has recently been published by Kumari and Dubey [15] on the cytotoxic effect of ethanolic extracts of *B. lupulina* on Hep G2 cell line. They found inhibitory effect of a single extract of *B. lupulina* on cancerous cells, Hep G2 and THP-1. Therefore, these differences caused the different results. Methanolic leaf extract of *Barleria strigosa* has been found to possess *in vitro* cytotoxicity against the P-388 murine leukemia cell line with CC_{50} of 413.89 µg/mL [24].

Anti-inflammatory activities of *B. lupulina* and *Clinacanthus nutans* extracts induced powerful dose-dependent inhibitory effects in both edema models in rats [2], who found a significant inhibition of myeloperoxidase (MPO) activity in the inflamed tissue indicating the association of anti-inflammatory effect of the extracts associated with reduced neutrophil migration. Although both extracts did not affect neutrophil viability or apoptosis, treatment of neutrophils with the extract concentration-dependently inhibited fMLP-induced chemotaxis, superoxide anion generation, MPO and elastase release. These findings suggest the powerful anti-inflammatory properties of *B. lupulina* and *C. nutans* extracts are mediated by inhibition of neutrophil responsiveness.

Apoptosis has been studied with the ROS production and mitochondrial membrane potential measurements. ROS production induced the intracellular damages including the DNA damage. During apoptosis, mitochondrial membrane became more permeable and thus released caspase and membrane activity was lost. In relation to

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cytotoxic effects and DNA fragmentation assay, *B. lupilina* extract induced ROS in the cells from 30 minutes of exposure. *B. lupilina* extract lowered the mitochondrial membrane potential significantly and thus inducing the apoptosis in the cells. The ethanolic leaf extract had potential effect on cancerous cell line.

Conclusion

It may be concluded that *B. lupulina* extract decreased in the cell viability of the cancerous cells. The present study also provides preliminary screening of this plant to have potent cytotoxicity against the cancerous cells. We believe that the reported improved method of DNA ladder assay will be very useful for numerous laboratories that routinely study cell death or carry out routine experimental/clinical screening of drugs and chemotherapeutics Therefore, it can be stated that *B. lupulina* may act as an anti-cancer drug.

Availability of data and materials

The data sets that support the conclusions of this article are included within the article.

Abbreviations: MPO - Myeloperoxidase; MMP - Mitochondrial membrane potential; ROS - reactive oxygen species; ELE - ethanolic leaf extract; NRU - Neutral red uptake; NCCS - National Centre for Cell Science; EMEM - Eagle's minimum essential medium; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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Authors' contributions

Conception, design, analysis and interpretation: RK and SK; drafting the manuscript for important intellectual content: RK and SK; final approval of the manuscript RK and SK. All approved the final manuscript.

Ethics declarations

NA

Consent for publication

NA

Competing interests

The author(s) declare that they have no competing interests.

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Figure 1. Different phyto-constituents of *B. Lupulina* ethanolic leaf exract at 366 nm on HPTLC plat with 10 µL of extracts.

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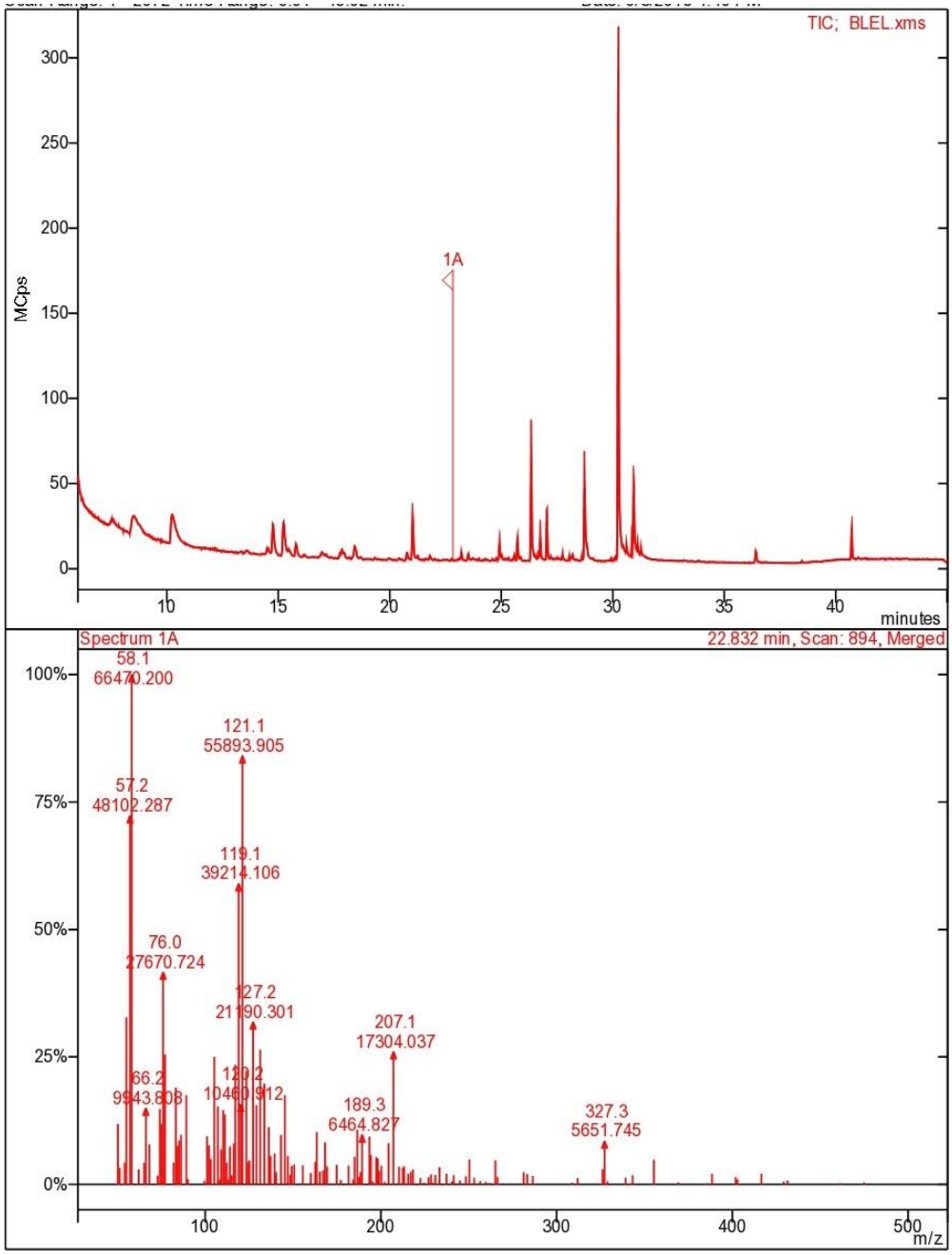

Figure 2. Chromatograph representing the peak areas of phyto-constituent of *B. Lupulina* ethanolic leaf extract matched with NIST library.

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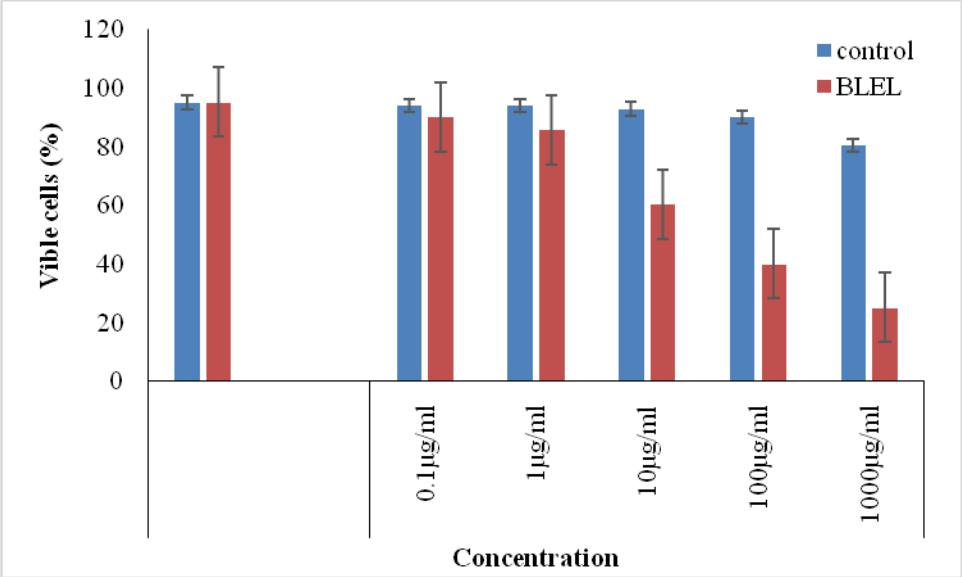

Figure 3. The bar diagram shows the effect of *B. lupulina* ethanolic leaf extract *in vitro* cytotoxicity against THP-1 cell. Data are expressed as the Mean ± SEM.

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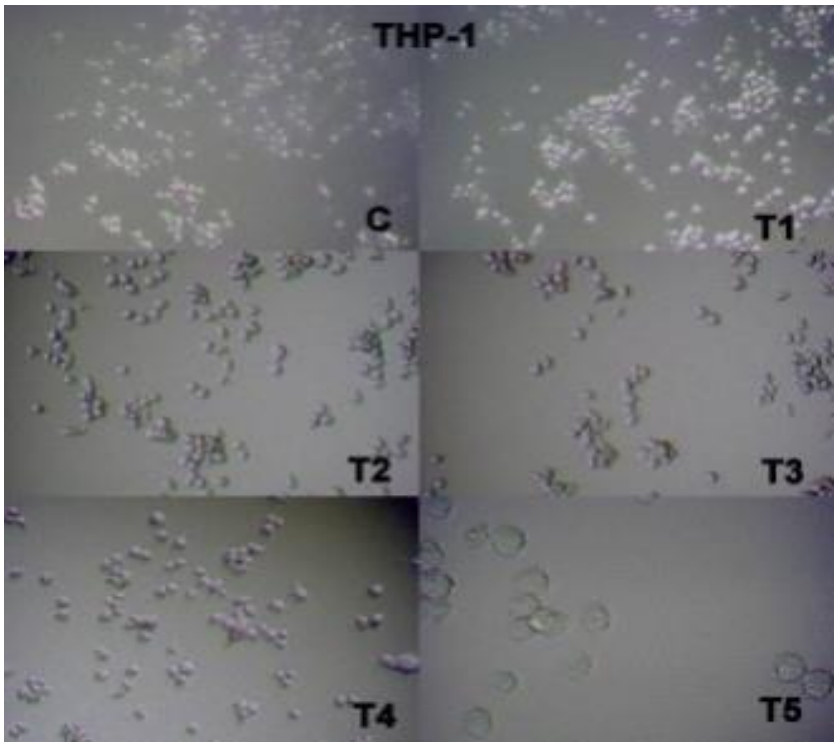

Figure 4. Cell viability and treatment of ethanolic leaf extract of *B. lupulina* on THP-1 cell line; (c) control cells; (T1) treatment 1; (T2) treatment 2; (T3) treatment 3; (T4) treatment 4; (T5) treatment 5.

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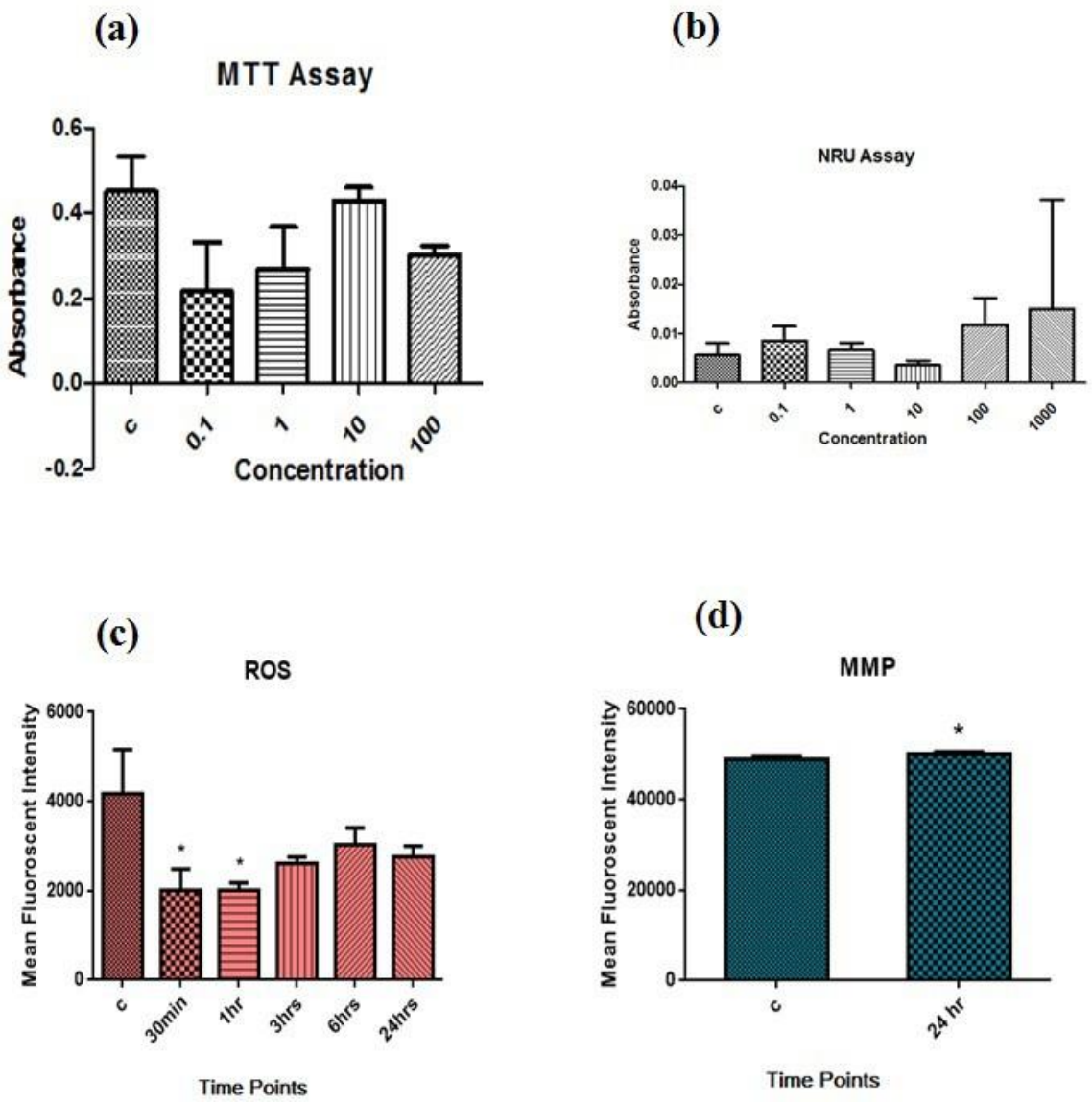

Figure 5. (a) Effect of ethanol soluble leaf extract of *B. lupulina* on live THP-1cell line with relative percentage using MTT assay (b) NRU assay (c) ROS production and (d) MMP measurements (in nm).

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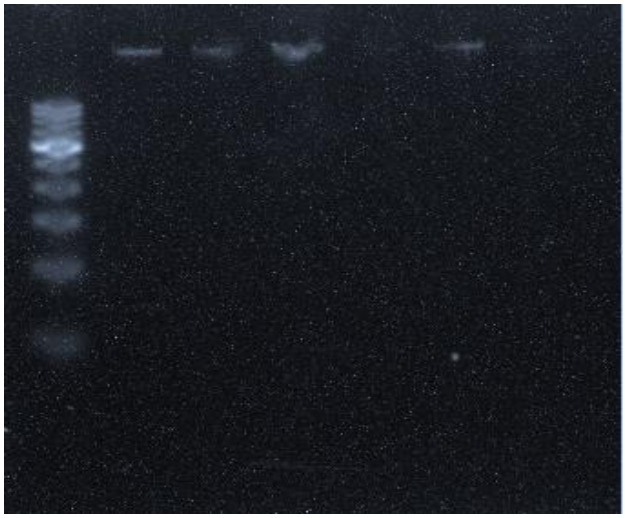

Figure 6. Showing DNA fragmentation of treated cells of THP-1.