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Induction of apoptosis by *Ocimum sanctum* (Holy basil) leaf extract against myeloid leukemia cells

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Abstract

Antineoplastic effect of *Ocimum sanctum* leaf extract was reported in our previous work. The cytotoxic potential of methanolic extract *Ocimum sanctum* (OSLE) was evaluated by MTT assay. Annexin-V FITC double staining was done to examine whether cell death occurred via apoptosis or necrosis. Gel electrophoresis, fluorescence microscopy, confocal microscopy and light microscopy were used to determine the cell morphology and fragmented DNA. JC-1 staining was also performed to measure the mitochondrial membrane potential. The phase distribution of the leukemia cell study was done by the cell cycle study. The pathway of apoptosis was studied by Caspase assay. OSLE significantly inhibited the cell viability in a time and concentration dependent manner in all the leukemia cells. After the treatment with OSLE, leukemia cells showed various signs of apoptosis like nuclear fragmentation, formation of apoptotic bodies, chromatin condensation and degraded DNA bands in cancer cells. The presence of apoptotic cells in the early and late stages was confirmed by the flow cytometric analysis. The cell cycle phase arrest was observed in the G0/G1 phase in the myeloid lymphoma and the multidrug resistant leukemia cells. A significant change in the mitochondrial membrane potential was observed in the OSLE treated leukemia cells with MMP shift assay. Caspase assay reveals that the pathway followed may be intrinsic apoptosis pathway. These finding suggest that the methanolic extract of *Ocimum sanctum* (OSLE) possesses anti-leukemic effects via apoptosis not necrosis along with insignificant toxicity.

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Introduction

Plants have served humans well as a valuable component of medicines, and have played a significant role in maintaining human health and improving the quality of human life for thousands of years. There is no doubt that increasing our intake of herbs is one of the most effective, convenient and economical ways in which we can fortify ourselves against infectious diseases and related cancers (Martins E, 2013). In the area of cancer prevention, plants consumption such as herbs and their constituents as potential chemopreventive agents remains an extensive research topic. Numerous studies have been published in regards to the relation between plants consumption, cancer prevention, antimicrobial effects, and overall protection of human health (Hanahan D. and Weinberg RA., 2011).

Ocimum species belonging to the family Lamiaceae is very important for its therapeutic potentials such as cardiopathy, leucoderma, asthma, haemopathy, bronchitis, vomiting, gastropathy, ring worm, verminosis and other skin disease etc. However, few chemical constituents and pharmacological activities have been reported on this species. Different parts of this plant have been reported to exhibit several medicinal properties. (Sheikh A., Yalavarthy P. D. *et al.,* 2016; P. Uma. Devi., 2001). Chemopreventive effects of extract of *Ocimum sanctum* have been observed in Human fibrosarcoma cells in culture (K. Karthikeyan, P. Gunasekaran *et al*., 1999), non-small cell carcinoma cell line (Sridevi M, Bright J *et al*., 2016), human breast cancer cell line, fibroblast cell line, etc. Studies have also shown that leaf extract of *Ocimum sanctum* is used to maintain hyperglycemia and complications associated with it in Type 2 Diabetes Mellitus (Bindu J. and Narendhirakannan R.T., 2019). Leukemia was not only reported as the 14th most common cancer but also the 11th leading cause of mortality worldwide. Also, it was ranked 8th and 9th in men and women respectively in terms of mortality. Overall, this disease accounts for 2.5% of the total cancer cases in people below the age of 75 years (Mohammadian M, Pakzad R, *et al., 2018*). Leukemia is the most common pediatric malignancy and a major cause of morbidity and mortality in children. In a patient with leukemia, many of the white blood cells produced in the bone marrow do not mature normally.

These abnormal cells, called leukemic cells, are unable to fight infection the way healthy white cells can. As they grow in number, the leukemic cells also interfere with the production of other blood cells (Gao P., Seebacher N. A., 2018). It is also characterized by the aggressive nature of the disease and poor response to therapy (Bob L and Jacob M. Rowe., 2016). Acute myeloid leukemia (AML) is the most common acute leukemia in adults (Graça M. D, Susan S. D *et al*., 2012). Sequencing of acute myeloid leukemia (AML) genomes revealed a predominance of DNA mutations occurring in genes related to transcription, chromatin regulation, and DNA methylation (Xianwen Y., Molly P., Man W. *et al.,* 2019). Chemotherapy and synthetic drugs lack specificity and targeted efficacy leading to drastic side effects. For thousands of years, humans have been using plants to treat various diseases (Lam S.N., Neda G.D. *et al.,* 2017). We have previously evaluated the effect of *Ocimum sanctum* methanol leaf extract (OSLE) against three types of colorectal carcinoma, one type of gastric adenocarcinoma, two types of hepatocellular carcinoma which showed anti-neoplastic activity in all the cells and in two types of normal cells which showed insignificant inhibition. Therefore, as a more rational approach, in this study we evaluated the effect of *Ocimum sanctum* leaf extract (OSLE) against U937, K562 and AML patient cells.

Materials and methods

Chemicals

RPMI1640 medium, Fetal Bovine Serum(FBS), Penicillin-streptomycin (Biowest, Germany), Gentamycin (Nicholas, India), HEPES, L- glutamine, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide)], Acridine orange, Ethidium bromide, RNAse, JC-1 dye, Propidium iodide (Sigma), Annexin V-FITC, Agarose (Puregene), Ethylene diamine tetra acetic acid (EDTA), Proteinase K (SRL), Caspase-8/FLICE Colorimetric Bio-Assay Kit (Bio Vision), Cat.K113-100, Caspase-9, Apoptosis Detection, Colorimetric Bioassay Kit (R&D Systems),

Catalog No.BF10100 and Caspase-3, Apoptosis Detection, Colorimetric Bio-Assay Kit (R&D Systems), Catalog No.BF3100, DMSO (dimethylsulphoxide), Chloroform, iso-amyl alcohol, Methanol (Merck), and all other chemicals and reagents were of analytical grade and procured locally.

Cell culture

Human leukemic cell lines U937 and K562 were purchased from National Facility for Animal Tissue and Cell Culture, Pune, India. Cells were routinely maintained inrpmI 1640 medium supplemented with 10% heat inactivated FCS. Cultures were maintained at 37℃ in a humidified atmosphere containing 5% CO2 in air. Blood from 20 untreated leukemic patients (age range 25–51 years) with a clinical diagnosis of AML (Ph+) were used in this study. Patients were from West Bengal, India. The peripheral blood was collected from the patients with informed consent. The peripheral blood of four normal individuals was also collected. From peripheral blood, only mononuclear cells were separated by Ficoll-Hypaque technique (Murmu N, Mitra S *et al.,* 2001). The cells were then taken inrpmI 1640 medium with 5% FCS in aseptic condition. In all the experiments untreated leukemic cells as well as untreated WBC were termed as control group.

Collection, extraction and preparation of test sample

The leaves of *Ocimum sanctum* were collected from the nursery of Ramakrishna Mission Ashram, Narendrapur. The plant was identified by Dr. K. Karthigeyan, scientist C, Central National Herbarium, Indian Botanical Garden, Howrah, India. Leaves of *Ocimum sanctum* were collected, shade dried and grinded into fine dust. 200gm of *Ocimum sanctum* leaf powder was taken in a conical flask and soaked in 500ml hexane for 3 days with occasionally shaking for removal of fat. After 3 days, the mixture was filtered and the filtrate was evaporated by Rotary evaporator. After evaporation 2gm of hexane extract was obtained and was stored in air tight container. After removal of fat from *Ocimum sanctum* leaves, it was soaked in 3x500ml of methanol for one week with occasional shaking. The mixture was filtered and the filtrate was evaporated by Rotary evaporator. After evaporation 3.4gm of sticky methanolic extract was obtained finally and designated as OSLE and kept in air tight container at 4°C. Stalk solution was prepared as 1mg/ml in PBS from here desired concentrations (25, 50, 100, 200µg/ml) was used for in-vitro experiments.

Cytotoxicity study by MTT assay

U937, K562 and AML patient cells ($1x10^5$) were separately incubated in 96-well sterile plates for 24, 48 and 72 hrs. All the treated cells with OSLE were grown in humidified atmosphere containing 5% CO2 in an incubator at 37℃ and the untreated cells were considered as control. After desired incubation 20µl of MTT (4-5mg/ml in PBS as a stock solution) was added to each well and incubated again for 3 to 4hrs at 37℃. The MTT assay is a colorimetric assay for assessing the metabolic activity of the cells or cell viability of NADPH dependent cellular oxidoreductase enzymes, and represents number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT, which is yellow in colour, to insoluble purple colored formazan. The intensity of the colour was measured at 490nm by micro-plate manager (Reader type: Model 680XR Bio-Rad Laboratories Inc.). The IC_{50} values were determined for the all the leukemia cells (Cao Z, Li Y, 2002).

Detection of cell morphology by light microscopy

U937 and K562 (1×10^6 cells) were treated with OSLE (IC_{50} dose 43.15µg/ml and 37.29µg/ml respectively) in a sterile Petri-plate and incubated for 24 h at the conditions mentioned above. The cells were then washed twice with PBS and observed under a light microscope (LeicaDMI4000B Microscope) at a magnification of 40X (Mishell BB, Shiigi SM, 1998).

Detection of cell morphology by fluorescence microscopy

U937 and K562 cells ($1x10^6$) were treated with IC_{50} of OSLE for 24 h were observed using a fluorescence microscope for morphological changes. The untreated control cells and the OSLE treated cells were harvested separately, washed with PBS and then stained with acridine orange (100µg/ml) and ethidium bromide (100µg/ml) (1:1). The cells were then immediately mounted on slides and observed

under a fluorescence microscope in (Olympus, Fluoview FV10i) at 60X for the morphological determination of the cells undergoing apoptosis.

Detection of cell morphology by confocal microscopy

U937 and K562 (1x10^6) were treated with 100µg/ml of OSLE for 24 h. After 24 h the untreated control cells and OSLE treated cells were harvested and washed with ice cold PBS. The cells were then stained with 10µg/ml of propidium iodide for 5 min. After mounting on slides the cells were observed to see the differences in nuclear morphology between the untreated and the OSLE treated leukemic cells under confocal laser scanning microscope (Olympus, Fluoview FV10i) installed with an inverted microscope as per Mishell et al's method. Images for propidium iodide was acquired from argon/krypton laser and UV laser line using 590nm long pass filter for propidium iodide and 450nm band pass filter for UV images. [2]

Detection of DNA fragmentation by agarose gel electrophoresis

U937 and K562 cells (1x10^6) were treated with IC_{50} dose of OSLE for 24 h. The cells were harvested and washed twice with PBS. The cells were resuspended in 500µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% SDS), 100µg/ml of proteinase K was added and incubation was done at 55°C for 1 h and 37°C overnight respectively. DNA extraction was done by following the general phenol-chloroform extraction procedure and kept at -20 °C overnight. After centrifugation, DNA precipitates were washed with 70% chilled ethanol, dried and evaporated at room temperature and dissolved in TAE buffer (pH 8.0) at 4°C overnight. To detect the DNA fragments, the isolated DNA samples were electrophoresed overnight at 20 V in 1% agarose gel and stained with ethidium bromide. DNA fragmentation was observed in UV transilluminator. (GENEI, Bangalore Genei Pvt. Ltd.) (Herrmann H, Lorenz HM *et al.,* 1994).

Detection of mitochondrial membrane potential (Δψm) assay

U937 and K562 (1x10^6) cells were treated with OSLE with desired dose and untreated as control for 24 hours to assay the mitochondrial membrane potential activity of cell in a flow cytometer. Cell were washed with PBS, pelleted down and eventually stained with JC-1 stain. The sample were incubated at 37°C for 15 min. Shift in the mitochondrial membrane potential was determined by FACS (Becton Dickinson FACS Fortessa 4 leaser cytometer), Fluorescence detector equipped with 520nm argon laser light source and 623nm band pass filter (liner scale) with the help of BD FACS Diva software (Becton Dickinson) (Nilanjana D., Anita H. *et al.,* 2017).

Detection of apoptosis by flow cytometric analysis

In order to investigate the type of cell death induced by OSLE, flow cytometric analysis was done by performing dot plot assay. U937 and K562 cells (1x10^6) were treated with desired dose of OSLE for 24 h. The cells were pelleted down, centrifuged at 2000rpm for 8 min at 4°C and washed with Annexin V FITC binding buffer (10 mM HEPES, 140 mM NaCl and 2.5mM CaCl2 2H2O; pH 7.4). Again after centrifuging at 2000rpm at 4°C, the cell pellets were dissolved in Annexin V FITC binding buffer containing annexin V FITC and propidium iodide. After 15 min incubation in dark at room temperature flow cytometric analysis was done. All data were acquired with a Becton Dickinson FACS Caliber single laser cytometer. Flow-cytometric reading was taken using 488nm excitation and band pass filters of 530/30nm (for FITC detection) and 585/42nm (for PI detection). Live statistics were used to align the X and Y mean values of the Annexin-V FITC or PI stained quadrant populations by compensation. Data analysis was performed with Cell Quest (Macintosh platform) program (Gupta SD, Debnath A *et al.,* 2007).

Detection of cell cycle arrest by flow cytometric analysis

In order to study the stage of cell cycle arrest in flow cytometry, U937 and K562cells (1x10^6) were treated with desired of OSLE for 18 h. Cells were washed with PBS, fixed with cold methanol by adding methanol drop-wise and kept at -20°C for 3 min. They were then resuspended in cold PBS and kept at 4°C for 90 min. Cells were pelleted down, dissolved in cold PBS, treated with RNase A for 30 min at 37°C and stained with propidium iodide (20µl from 50µg /ml) and kept

in dark for 15 min. Cell cycle phase distribution of nuclear DNA was determined on BD FACS Diva software (Becton Dickinson FACS) (Gupta SD, Debnath A *et al.,* 2007).

Caspase-8 Assay

The assay was performed using a Caspase-8, Apoptosis Detection, Caspase-8/FLICE Colorimetric Bio-Assay Kit (Bio Vision), Cat.K113-100 according to the manufacturer's protocol. U937 cells ($1x10^7$) were treated with IC_{50} dose for 24 h. The cells were pelleted down and resuspended in 50µl of cell lysis buffer (supplied with the kit) and incubated on ice for 10 min. After centrifuging at 10,000 x g for one min, the supernatants (cytosolic extract) were transferred to fresh tubes and kept on ice and the caspase-8 assay was performed according to the supplied kit protocol. 50µl of 2X reaction buffer (containing 1M DTT) was added to each sample. 5µl of IETD-pNA substrate (4 mM) (200 µM final concentration) was added and incubation was done at 37°C for 1-2 h. Absorbance was read at 405nm and calculations were thereby done (Shamik B., Tanaya D. *et al.,* 2013).

Caspase-9 Assay

The assay was performed using a Caspase-9, Apoptosis Detection, Colorimetric Bioassay Kit (R&D Systems), Catalog No.BF10100 according to the manufacturer's protocol. U937 cells ($1x10^7$) were treated with IC_{50} dose for 24 h. The cells were pelleted down and resuspended in 50µl of cell lysis buffer (supplied with the kit) and incubated on ice for 10 min. After centrifuging at 10,000X g for one min, the supernatants (cytosolic extract) were transferred to fresh tubes and kept on ice and the caspase-9 assay was performed according to the supplied kit protocol. 50µl of 2X reaction buffer (containing 1M DTT) was added to each sample. 5µl of LEHD-pNA substrate (4 mM) (200 µM final concentration) was added and incubation was done at 37°C for 1-2 h. Absorbance was read at 405nm and calculations were thereby done (Piyali B and Anupam B, 2013).

Caspase-3 Assay

The assay was performed using a Caspase-3, Apoptosis Detection, Colorimetric Bio-Assay Kit (R&D Systems), Catalog No.BF3100 according to the manufacturer's protocol. U937 cells ($1x10^7$) were treated with IC_{50} dose for 24 h.

The cells were pelleted down and resuspended in 50µl of cell lysis buffer (supplied with the kit) and incubated on ice for 10 min. After centrifuging at 10,000 x g for one min, the supernatants (cytosolic extract) were transferred to fresh tubes and kept on ice and the caspase-3 assay was performed according to the supplied kit protocol. 50µl of 2X reaction buffer (containing 1M DTT) was added to each sample. 5µl of DEVD-pNA substrate (4 mM) (200 µM final concentration) was added and incubation was done at 37°C for 1-2 h. Absorbance was read at 405nm and calculations were thereby done (Piyali B. and Anupam B., 2013).

Statistical analysis

Percentage of cell growth inhibition was calculated by the following formula:% Cell Inhibition= 10 X (O.D of Control –O.D. of treated /O. D. of Control), O. D= Optical Density. Percentage of cell viability was calculated as follows: Viable Cells (%) = (Total number viable cells perml/Total number of cells per 1ml) x100.

Results

Cytotoxicity study by MTT assay

Treatment with OSLE by MTT assay showed significant reduction in the O.D values in the leukemia cells U937, K562, PBMC of AML patient cells in a time and concentration dependent manner. These observations provided proof for cytotoxic nature of OSLE towards only on all the leukemia cell lines. The IC_{50} value of OSLE treatment for 24hrs was calculated to be 43.15µg/ml for U937 Acute myeloid leukemia cell line, 37.29µg/ml for K562 chronic leukemia cell line (multi drug resistant) and 36.57µg/ml for PBMC of AML cell line.

Detection of cell morphology by light microscopic studies

Light microscopic images of U937 and K562 cells treated with OSLE (**I**C_{50} dose) revealed the presence of fragmented nuclei as compared to the untreated control cells with intact nuclei.

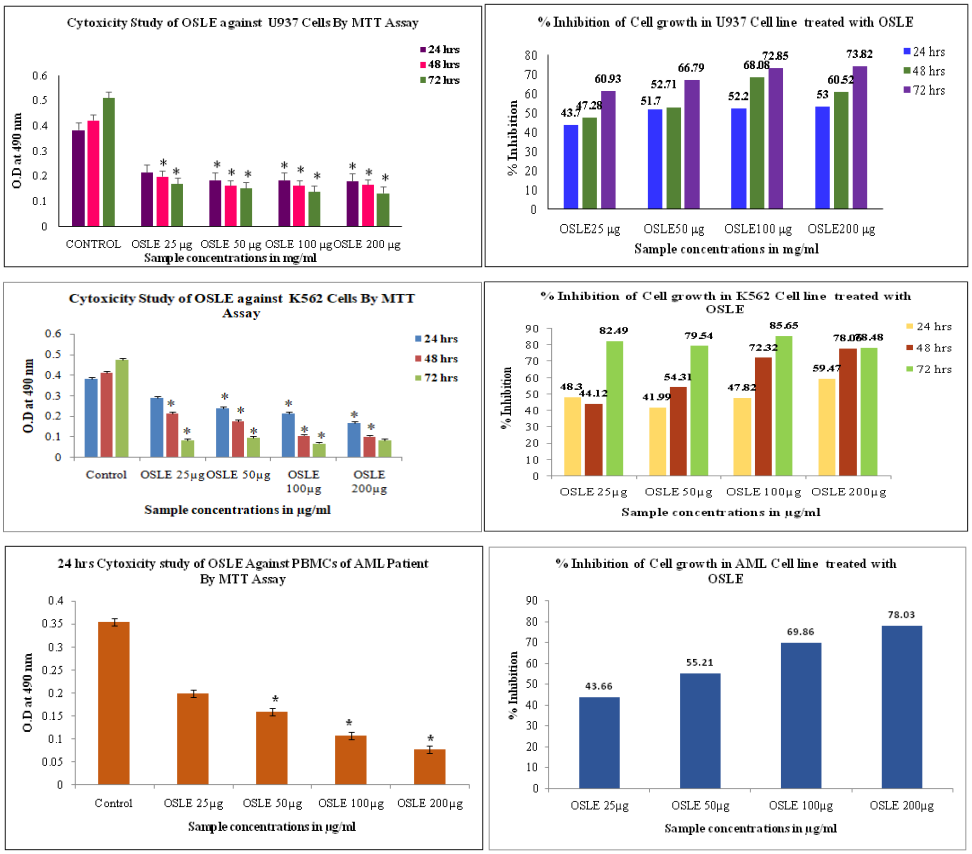

Fig. 1. Histogram shows the O.D values and the% Inhibition of OSLE on cell viability of U937 and K562 cell line after 24, 48, 72 hrs of treatment respectively and of PBMC of AML cell line after 24 hrs By MTT Assay. OSLE treated all the leukemia cells showed significant reduction in O.D. value and significant increase in% inhibition in a time and concentration dependent manner. The IC_{50} value of OSLE were calculated for U937 43.15µg, K562 37.29µg and PBMC of AML 36.57µg in 24 hrs. p value is less than 0.05. Data are mean ±S.E.M.* Denote a significant decrease in O. D. at 490nm from control values $P<0.05$.

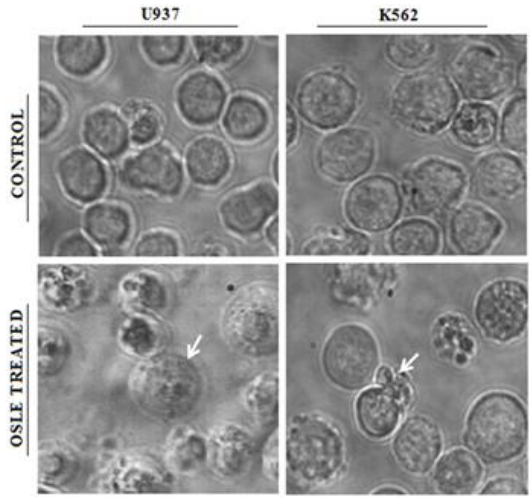

Fig. 2. Light microscopic images of U937 and K562 cells showed untreated control cells with intact nuclei, methanolic extract of *Ocimum Sanctum* leaves (OSLE) treated cells with fragmented nuclei showing signs of apoptosis**,** shown by the arrowheads.

Detection of cell morphology by fluorescence microscopic studies

U937, K562, PBMC of AML patient cells were treated with OSLE and stained with both acridine orange and ethidium bromide. OSLE treated cells confirm the presence of early and late apoptotic cells as compared to the untreated control cells. In this study nuclear changes were observed including chromatin condensation and apoptotic body formation that are the indication of apoptotic processes.

Detection of cell morphology by Confocal microscopic studies

OSLE induced apoptotic changes in U937 and K562 and cells after 24 h of treatment showing chromatin disintegration and formation of apoptotic bodies whereas the untreated control cells were with intact nuclei.

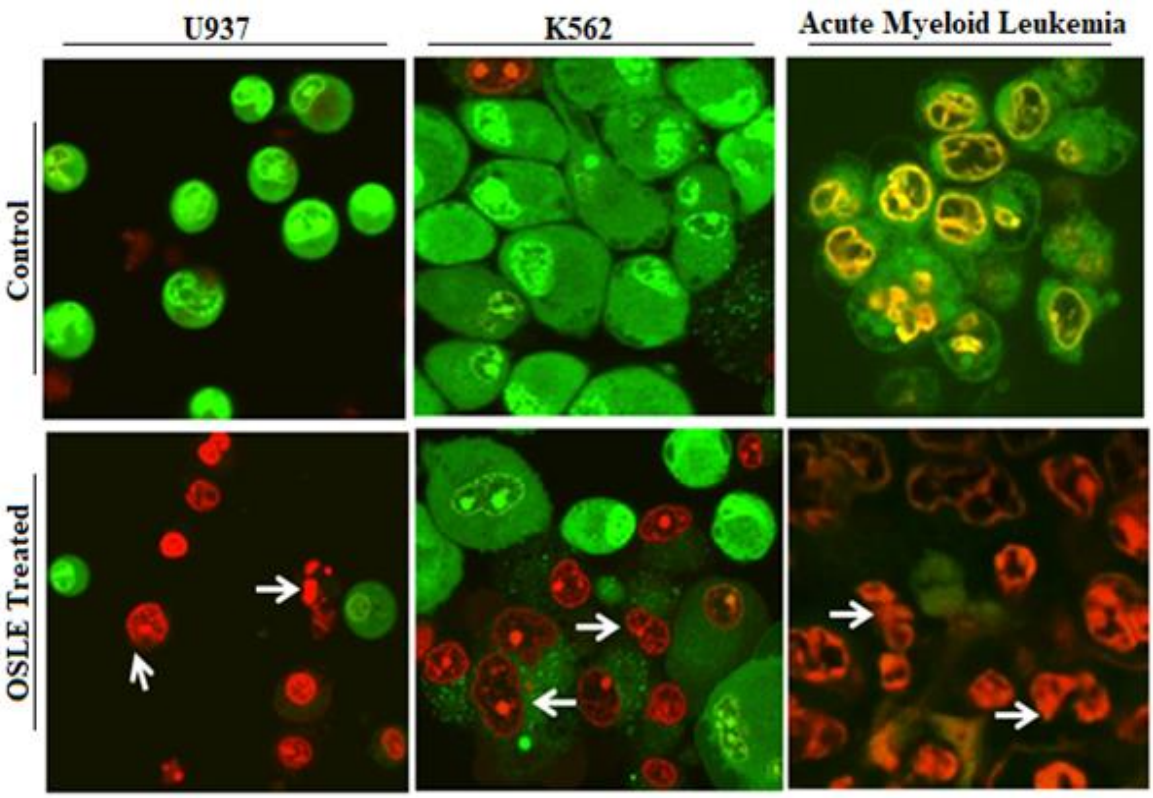

Fig. 3. Fluorescence microscopic images of both the untreated control and OSLE treated U937, K562 and PBMCs of AML patients cells with IC_{50} dose. The control cells give a bright green fluorescence whereas the treated cells show an orange-red color, demarking the occurrence of Apoptosis in U937, K562 and PBMCs of AML patient cells.

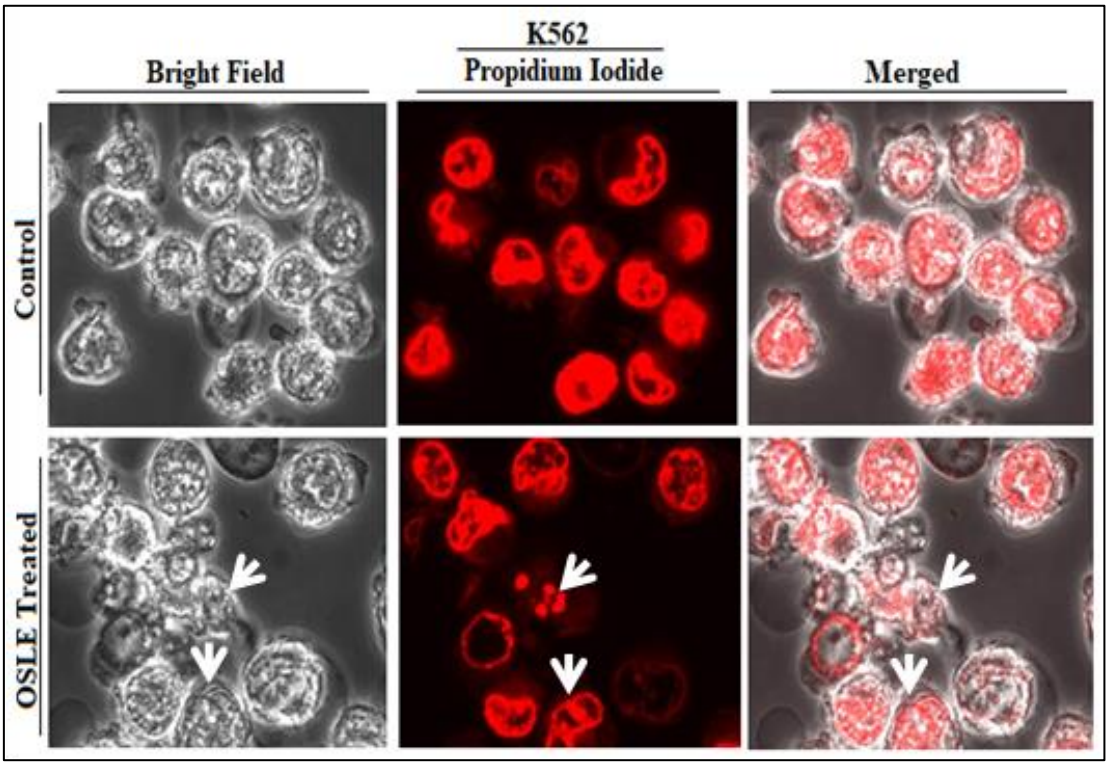

Fig. 4. Confocal microscopic images of untreated control U937 and K562 and treated U937 and K562 cells. The control cells were with intact nuclei whereas OSLE treated cells indicated apoptotic changes like nuclear disintegration and formation of apoptotic bodies, shown by the arrowheads.

Detection of DNA fragmentation by agarose gel electrophoresis

Agarose gel electrophoresis of the DNA samples isolated from untreated and control U937 and K562 cells showed intact DNA bands, whereas OSLE treated U937 and K562 cells showed degraded DNA bands in the form of ladders. So, the observations confirmed that the treatment with OSLE in both the cells caused apoptosis.

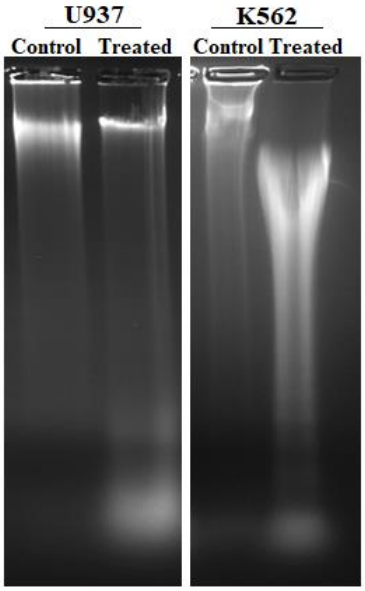

Fig. 5. DNA fragmentation by agarose gel electrophoresis in U937 and K562 cells. Lane 1 represent control cells which shows intact DNA whereas Lane 2 represent OSLE desired dose treated cells in which DNA fragments are clearly visible in Leukemia cells.

Detection of mitochondrial membrane potential (Δψm) assay

Mitochondrial dysfunction is an essential target for induction of apoptosis. The Human acute myeloid leukemia cell line (U937) and multidrug resistant cell line (K562) cells lines when treated with desired dose of OSLE, showed a loss of Mitochondrial Membrane Potential (Δψm). The JC-1 stain cannot accumulate in the mitochondria of the apoptotic cells, as the mitochondrial membrane potential collapses, hence showing green fluorescence (P4) denotes apoptotic cells and red fluorescence (P3) denoting healthy cells where JC-1 stain accumulates. Depolarization in mitochondrial membrane potential was observed by staining untreated and treated cell by JC-1 dye. The depolarization led to a transmembrane shift from red to green fluorescence leading to release of cytochrome c. A significant transmembrane shift of 2.2% to 9.8%, 19.0% to 59.2% were observed when U937 and K562 cells were treated with the IC_{50} value of OSLE for 24 hours respectively.

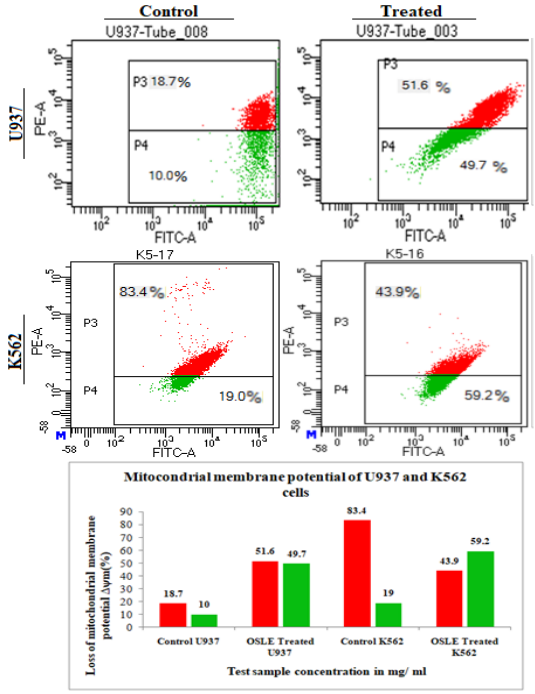

Fig. 6. Flow cytometric analysis of mitochondrial membrane potential (Δψm) on U937 and K562 cell lines in both control and OSLE treated cells respectively after 24 hrs of treatment. Transmembrane shift significantly increased in U937 (49.7% in treated from 10% in control) and in K562 cells (59.2% in treated from 19% in control) when treated with desired dose of OSLE from red to green fluorescence was observed.

Detection of apoptosis by flow cytometric analysis

In the flow cytometric analysis, double labeling technique, using Annexin V-FITC and propidium iodide, was utilized. Lower left (LL) quadrant (Annexin V-/PI-) is regarded as the population of live cells, lower right quadrant (LR) (Annexin V+/PI-) is considered as the cell population at early apoptotic stage, upper right (UR)quadrant (annexin V+/PI+) represents the cell population at late apoptotic stage. Flow cytometric data analysis revealed that after 18 h

of treatment with desired dose of OSLE for quantification of apoptosis was observed for U9370.4% against 38.3% and for K562 8.3% against 89.6% cells were in upper right quadrant which implies apoptotic cells thereby, showing apoptotic inducing property of OSLE on Human acute myeloid leukemia and multi drug resistant cells.

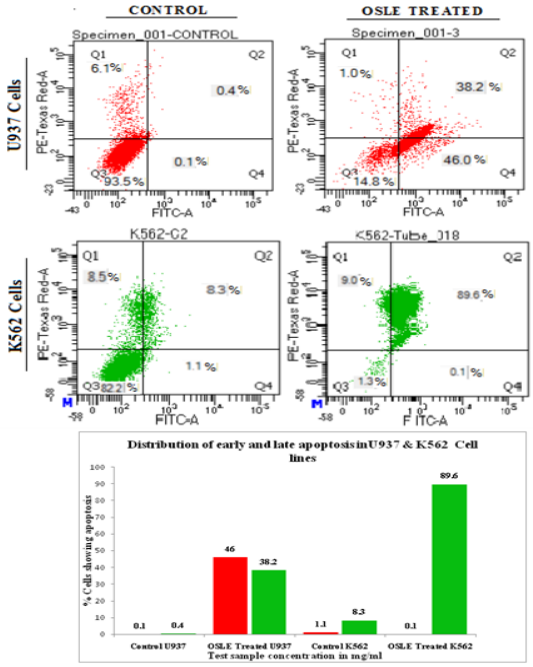

Fig. 7. Flow cytometric analysis of untreated control and OSLE treated U937 and K562 cells respectively stained with Annexin V FITC and propidium iodide. Dual parameter dot plot of FITC fluorescence (x-axis) vs PI-fluorescence (y-axis) shows logarithmic intensity.

Detection of cell cycle arrest by flow cytometric analysis

Flow cytometry analysis showed that after 24hrs treatment of U937 and K562 cell lines with OSLE at desired dose, sub-G1 peak was markedly changed. The DNA content increased in OSLE treated cell 53.4% against 47.7% in U937, 54.0% against 45.8% in K562cells. These observations revealed that OSLE significantly inhibited the growth of U937 and K562 leukemia cells by arresting the cell populations in the sub-G0/G1 phase of the cell cycle.

Caspase-8 Assay

Caspase-8 Assay analysis showed that after 24hrs treatment of U937 cell lines with OSLE at desired dose, the Caspase-8 activity did not change markedly. The Caspase-8 activity changed negligibly in OSLE treated cells by 0.12 fold in U937 cells. This observation revealed that OSLE induced apoptosis may not follow extrinsic apoptotic pathway.

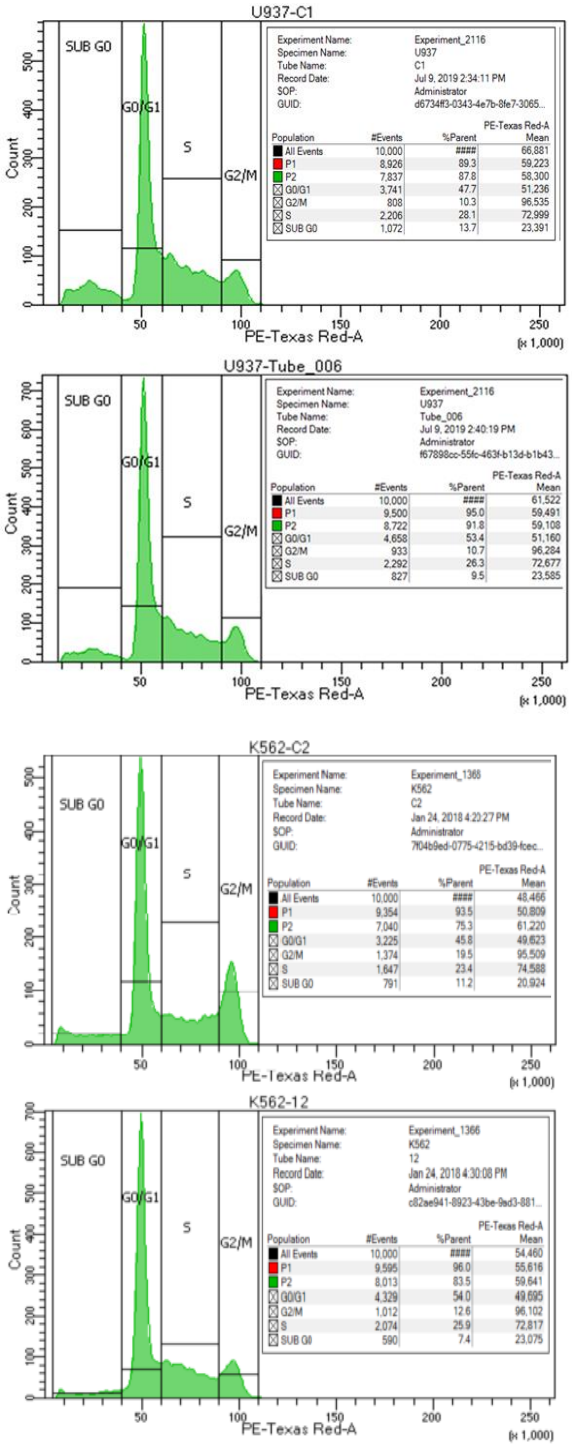

Fig. 8. Flow cytometric analysis of cell cycle phase distribution in control and treated of U937 and K562 cells respectively after 18 hrs treatment at desired dose of OSLE. Histograms represent various contents of DNA with actual number of cells.

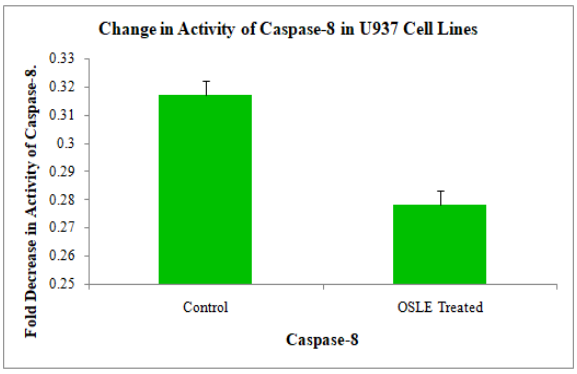

Fig. 9. Caspase-8 Assay analysis in control and treated U937 cells respectively after 24 hrs of treatment at desired dose of OSLE. Histogram represent change in activity of treated with respect to the control with actual number of cells.

Caspase-9 Assay

Caspase-9 Assay analysis showed that after 24hrs treatment of U937 cell lines with OSLE at desired dose, the Caspase-9 activity markedly changed. The Caspase-9 activity increased in OSLE treated cells by 2.31 fold in U937 cells. These observations revealed that OSLE induced apoptosis may follow intrinsic apoptotic pathway rather than extrinsic.

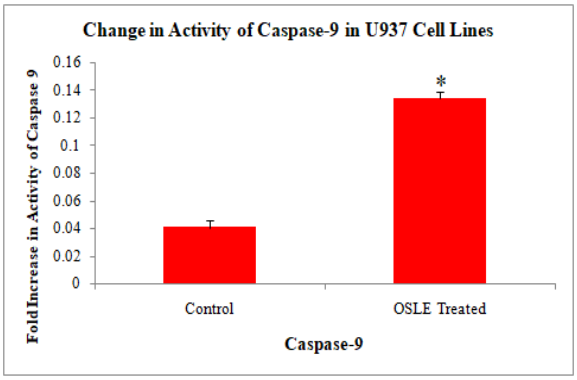

Fig. 10. Caspase-9 Assay analysis in control and treated U937 cells respectively after 24 hrs treatment at desired dose of OSLE. Histogram represent increase in activity of treated with respect to the control with actual number of cells.

Caspase-3 Assay

Caspase-3 Assay analysis showed that after 24hrs treatment of U937 cell lines with OSLE at desired dose, the Caspase-3 activity changed markedly. The Caspase-3 activity increased in OSLE treated cells by 2.93 fold in U937 cells. This observation revealed that OSLE induced apoptosis may follow intrinsic apoptotic pathway.

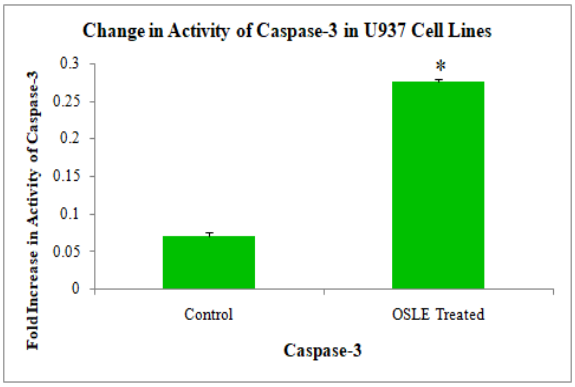

Fig. 11. Caspase-3 Assay analysis in control and treated U937 cells respectively after 24 hrs treatment at desired dose of OSLE. Histogram represent change in activity of treated with respect to the control with actual number of cells.

Discussion

Cancer is a group of diseases involving abnormal cell growth which is caused by mutation of genes involved in the cell division and its control. This mutation can either accelerate the rate of cell division or inhibit the normal control on the system like arrest of cell cycle or apoptosis. It has the potential to invade or spread to other parts of the body (Roy S., Besra S. E. *et al.,* 2008). In the most developed countries and the developing countries, cancer is among the three most common causes of death.

Treatment of cancer involves chemotherapy, radiotherapy, surgery and often a combination of two or three (Singh N., Verma P. *et al.,* 2012). The lack of specificity and targeted efficacy of chemotherapy and synthetic drugs leads to drastic side effects. Humans have been using plants to treat various diseases for thousand years. The active anticancer agents have been identified from many plants whose potency to inhibit the cancer metastasis is studied extensively (Houghton P, Fang R *et al.,* 2007).

It is shown that once a plant extract is found to possess anti-inflammatory activity, the test for anti-cancer activity should also be done as it is well established that inflammation and cancer go hand in hand (Siti M. A., Ibrahim J. *et al.,* 2018).

Ocimum sanctum L. (also known as *Holy Basil*, Tulsi) has been used for thousands of years in Ayurveda for its diverse healing properties. Tulsi (incomparable one of India), is one of the holiest of all the herbs which is renowned not only for its religious and spiritual sanctity but also for its important role in the traditional Ayurvedic and Unani system of herbal medicine of the East (Besra SE, Sharma RM *et al.,* 1996). Research has indicated that Tulsi reduces inflammation, prevents gastric ulcers, protect from radiations, lowers cholesterol and pressure, antibacterial and antifungal activity, etc.

Since the anti-cancer activity of aqueous methanolic extract of *Ocimum Sanctum* leaf (OSLE) extract has already been reported in three types of colorectal carcinoma, one type of gastric adenocarcinoma and two types of hepatocellular carcinoma by using SW480, HCT116, HT29, AGS, Huh-7 and HepG2 cell lines respectively (PriyabrataP, PritishovaB *et al., 2010),* its anti-leukemic activity has been evaluated against three human leukemic cell lines- U937, K562, and PBMC of AML patient cells in the present investigation. The cytotoxic, apoptotic and anti-proliferative activities of OSLE were supported by the observations in MTT assays and apoptotic studies respectively. OSLE not only inhibited growth but also induced apoptosis in U937, K562 and PBMC of AML patient cells in a concentration-dependent manner. In previous study it was also found that at concentrations used in present study it did not show any cytotoxic effect in macrophage cell line, RAW264.7 and in normal human embryonic kidney cell line HEK293T as observed in MTT assay. These findings reveal that OSLE possess anti-leukemic activity (Gaurav A., Nilanjana D. *et al.,* 2019).

Anti-leukemic effect of OSLE was established by morphological studies like light microscope, fluorescence microscope, and confocal microscope. Apoptotic cells are characterized by morphological changes such as membrane blebbing, chromatin condensation, cell shrinkage, nuclear fragmentation and formation of apoptotic bodies. Fluorescence microscope images of OSLE treated U937, K562 and PBMC and AML cells showed nuclear disintegration compared to that of the untreated control cells stained with acridine orange and ethidium bromide. The untreated control cells showed bright green fluorescence with intact membrane. Whereas OSLE treated leukemia cell lines showed intense orange-red fluorescence reduced green fluorescence. Further, the gel patterns of agarose gel electrophoresis of OSLE treated leukemia cells support the anti-leukemia activity of OSLE. The OSLE treated leukemia cells showed degraded DNA bands in the form of ladders, evidence of apoptosis, whereas intact DNA bands was showed in untreated control cells when observed in UV transilluminator. Marked increase in apoptotic cells in OSLE treated U937 and K562 cell line than control cells was seen in mitochondrial membrane potential assay by JC-1 dye. The transmembrane shift from red to green fluorescence due to depolarization of mitochondrial membrane leading to release of cytochrome C. There was also increase number of cells in the early and late apoptotic stage as shown in the experiments after treatment with OSLE supporting the fact that apoptosis was triggered by the treatment with OSLE in U937 and K562 leukemia cells. It was also revealed in the Cell cycle analysis that the treatment with OSLE arrested the cell populations in the sub G_0/G_1 phase of the cell cycle whereas in the previous study is has been shown that that OSLE insignificantly inhibited the cell growth in both the normal human embryonic kidney cell lines (HEK293T) and murine macrophage cell line (RAW 264.7) cell lines implying the fact that OSLE donot change the cell populations as compared to control cells of cell cycle. Mitochondrial Membrane potential studies also revealed that treatment with OSLE donot trigger apoptosis in both the normal cells.

Conclusion

From the experimental studies carried out with the methanolic extract of leaves of *Ocimum sanctum,* it can be concluded that the methanolic extract of OSLE have anti-leukemic activity on acute myeloid leukemia cell line (U937), multidrug resistant cell line (K562) and on PBMC of AML patient cell line. Therefore, it can be stated that leaves extract of *Ocimum sanctum* could be a probable and potent sources of anti-cancer agent for the treatment of

leukemia with less side effects. Further study should be done to identify the active compound/compounds of *Ocimum sanctum* plant which are responsible for anti-leukemic/anticancer activity on cancer cells.

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Conflict of interests

The authors declare that they have no conflict of interests.

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