Introduction

Leukemia is a type of cancer in which blood cells proliferate abnormally and uncontrollably in the bone marrow because of epigenetic and genetic changes. In this disorder, the hematopoietic stem cell produces more immature WBC, which is then replaced by normal WBC, resulting in an immune-compromised condition. In developed countries, Leukemia is one of the most common cancers among persons over the age of 55, but it is also the most common cancer in children younger than 15. Fatigue, weight loss, frequent infections, and easy bleeding or bruising are all symptoms of rapidly growing types of Leukemia. Several therapy techniques have been used in recent years to combat the complexity of various cancers. Chemotherapy is the only effective treatment for malignant Leukemia. The most serious disadvantage of the presently available effective artificial anti-cancer medications is their toxicity, various side effects, and reappearance of symptoms after treatment. One of the most significant challenges in chemotherapy is drug resistance. It is necessary to use natural methods for the treatment of leukaemia to avoid this problem. Natural-derived compounds are gaining popularity among scientists as they have less severe side effects than conventional treatments like chemotherapy. Murraya paniculata is used in traditional medicine as an anti-inflammatory, antimicrobial, and analgesic. Antimicrobial properties of alkaloids and flavonoids found in the leaves and stems of M. paniculata can be used to treat toothaches. The whole plant’s methanolic extract includes a substantial amount of flavonoids and has excellent antioxidant effects. So, we evaluate the effect of methanolic extract of Murraya paniculata leaves (MEMPL) against human myeloid Leukemia (U937) cell line.

Materials and Methods

Chemicals and Reagents

RPMI 1640 medium with L-glutamine, Fetal Calf Serum (FCS), HEPES, Histopaque 1119, Ethidium bromide and Acridine orange, Propidium iodide were purchased from Sigma (St. Louis, MO, USA), Penicillin-Streptomyacin (BioWest, Germany), Gentamycin (Nicholas, India), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Dimethyl sulfoxide (DMSO), Annexin V FITC, RNAse, JC-1 (sigma), Ethylene Diamine Tetra acetic Acid (EDTA) (Sisco R.L Pvt. Ltd), Sodium bi-carbonate & Ethanol (Merck) and other chemicals and reagents were of analytical grade and purchased from local firm.

Cell Culture

Human leukemic cell lines U937 were purchased from National Facility for animal tissue and cell culture, Pune, India and supplied from Indian Institute of Chemical Biology for in-vitro studies. Leukemic cells were sub cultured weekly at an initial concentration of 1×10⁶ cells/ml and maintained in sterile RPMI 1640 medium supplemented with 10% heat inactivated FCS. Culture was

ABSTRACT

We evaluated the cell viability activity of methanolic extract of Murraya paniculata leaf (MEMPL) on leukemia cells. MEMPL significantly reduced the cell viability in a time and concentration dependent manner in both the acute myeloid and acute lymphoid cells. Several apoptosis indicators were found in cancer cells, including chromatin condensation, nuclear fragmentation according to the morphological study. These findings suggest that MEMPL exhibited anticancer activity in leukemic cell lines. Flow cytometric analysis found a significant number of cells in the early and late stages of apoptosis. MMP shift assay revealed a significant change in mitochondrial membrane potential in MEMPL-treated leukemic cells. The cells were mostly in the G0/G1 phase of the cell cycle when they were arrested. These findings reveal that MEMPL showed anticancer activity in leukemic cell lines.

Keywords: Murraya paniculata, Leukemia, Myeloid, Lymphoid, Apoptosis.

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maintained at 37°C in a humidified atmosphere containing 5% CO₂ in air. In all experiments untreated leukemic cells were termed as control group.

Collection and isolation of AML patient cell

Fifteen patients with a clinical diagnosis of AML were analysed in this study and human ethical clearance was taken from institutional and hospital ethical committee. All the patients were from E.S.I. Hospital Sealdah, 301/3, Acharya Jagadish Chandra Bose Rd, Raja Bazar, Kolkata, West Bengal 700009, India. They were newly diagnosed and had not undergone any treatment. Informed consent was obtained from all the patients before sampling. Peripheral blood was obtained from which mononuclear cells were isolated by density gradient centrifugation (400g interphase, 20 min) with Histopaque (Sigma). After isolation the cells were washed with PBS and kept in RPMI medium with 10% heat inactivated FCS. Culture was kept at 37°C in a humidified atmosphere containing 5% CO₂ in air for experiment.

Collection and Identification of Test Sample

*Murraya paniculata* (L.) Jack leaves were collected from Howrah, West Bengal during March 2014. The plant was identified by Dr. K. Karthigeyan, Scientist C, Central National Herbarium, Botanical Survey of India, Howrah - 711109 where a specimen was deposited for identification.

The identification number is No: CNH/42/2014/Tech.II/108.

Extraction and Preparation of Test Sample

*Murraya paniculata* leaves were air dried, grinded into powder (180 gm) and soaked in just about 350ml petroleum ether in room temperature for 2 times. From that process, Petroleum ether extract was obtained. It was then dissolved in 250 mL of chloroform and kept for 48 hours. The Chloroform extract was produced after repeating this process two times. Then, it was further dissolved in 250ml of methanol in room temperature for 7 days with random shaking. The mixture was then filtered using filter paper, the filtrate was evaporated using a Rotary Vacuum Evaporator, and the extract was lyophilized for four days to get a methanol-free extract. Finally, the sticky methanicolic extract was obtained. It was kept in airtight container and sealed with para film. Finally, the container is stored in 4°C and designated as methanicolic extract of *Murraya paniculata* leaf extract (MEMPL) for the experiments. Stock solution was prepared (3mg/ml concentration) in phosphate buffer saline (PBS) from which desired doses were tested.

Cytotoxicity Study by MTT Assay

To evaluate the cytotoxicity activity of MEMPL on Peripheral blood mononuclear cell line (PBMCs) and human myeloid Leukemia cell line (U937) by performing the MTT assay, all the cells (1x10⁶) were seeded in 96- well sterile plates separately and were treated with different concentrations (25, 50, 100 μg/ml) of MEMPL for 24 hrs All the cells were developed in humidified atmosphere containing 5% CO₂ in an incubator at 37°C for 24 hrs. The untreated cells were considered as control. In incubation, 20µl of MTT (4-5mg/ml in PBS as a stock solution) was added to each well and incubated again for 4hrs at 37°C after 24 hrs. The MTT assay is a colorimetric assay for determining cell metabolic activity or cell viability of NAD(P)H-dependent cellular oxidoreductase enzymes, and it indicates the number of viable cells present. These enzymes can transform the yellow tetrazolium dye MTT to the insoluble purple coloured formazan. The intensity of the colour was measured at 570 nm by micro-plate manager (Reader type: Model 680 XR Bio-Rad Laboratories Inc.). The IC₅₀ values can be determined for the different cells from the O.D value.

Fluorescence Microscopy Study

U937 cells (1x10⁶) were treated with IC₅₀ of MEMPL for 24 hrs. and observed using a fluorescence microscope for morphological changes. The MEMPL-treated cells and the untreated control cells were observed separately, washed in PBS, and stained with acridine orange (100 g/ml) and ethidium bromide (100 g/ml) (1:1). The cells were then put on slides and studied under a fluorescence microscope to evaluate the morphology of the apoptotic cells.

Mitochondrial Membrane Potential Study

In a flow cytometer, U937 (1x10⁶) cells were treated with MEMPL at the required dose and left untreated as a control for 24 hours to evaluate the mitochondrial membrane potential activity of the cells. The cells were washed with PBS and pelleted, then stained with the JC-1 dye. The sample was incubated at 37°C for 15 minutes. Mitochondrial membrane potential changes were revealed by FACS (Becton Dickinson FACS Fortessa 4 leaser cytometer), Fluorescence detector equipped with 570 nm argon laser light source and 623 nm band pass filter (liner scale) with the BD FACS Diva software.

Detection of apoptosis by flow cytometric analysis

Flow cytometric analysis was done by performing dot plot assay, U937 cells (1x10⁶) were treated with desired dose of MEMPL for 24 h to investigate the type of cell death induced by MEMPL. 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). The cell pellets were dissolved in Annexin V FITC binding buffer containing annexin V FITC and propidium iodide after centrifugation at 2000rpm at 4°C. Flow cytometric analysis was done after 15 min incubation in dark at room temperature. All data were obtained with a Becton Dickinson FACS Caliber single laser cytometer. Flow-cytometric reading was done using 488nm excitation and band pass filters of 530/30nm (for FITC detection) and 585/42nm (for PI detection). By compensation, the Annexin-V FITC or PI-stained quadrant populations’ X and Y mean values were aligned using live statistics. The Cell Quest programme (Macintosh platform) was used to analyze the data.
Study of Cell Cycle Arrest by Flow Cytometry

U937 cells were treated with MEMPL (IC_{50} dose) for 24 hours to determine the stage of cell cycle arrest in flow cytometry. PBS was used to wash the cells and cold methanol was used to fix them. After that, they were resuspended in cold PBS and stored at 4°C for 90 minutes. Cells were pelleted, dissolved in cold PBS, treated in RNase at 37 °C for 30 minutes and stained with Propidium iodide, and stored in the dark for 15 minutes.18 The cell cycle phase distribution of nuclear DNA was discovered on FACS (Becton Dickinson FACS Fortessa 4 laser cytometer), fluorescence detector with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using BD FACS Diva software (Becton Dickinson).

Statistical Analysis

Student’s t test was used for statistical analysis and p<0.005 was considered significant.

The percentage of cell growth was calculated by the following formula:

\[ \% \text{ Cell inhibition} = 100 \times \frac{\text{O.D of control} - \text{O.D of the treated}}{\text{O.D of control}} \]

Where, O.D refers to Optical density

The percentage cell viability was calculated by the formula:

\[ \text{Viable cells} (\%) = \frac{\text{Total number of viable cells per ml}}{\text{Total number of cells per ml}} \times 100 \]

RESULTS

Cytotoxicity Study

In the cytotoxicity study by MTT assay, there was significant reduction in the O.D values after treating the AML patient’s cells and U937 cells with MEMPL in a time and concentration dependent manner compared to the control cells. These observations provide proof for cytotoxic nature of MEMPL on these cells. Therefore, IC_{50} dose of MEMPL was 27.64 µg for U937 cell line and 47.59 µg for AML patient’s cells.

Figures 1 and 2: Histograms showing the cytotoxicity of MEMPL on AML patient’s cell and U937 cell after 24hrs by MTT assay. Reduction in the O.D at 570 nm is observed in a time and dose dependent manner. Data are mean ± S.E.M. * denotes significant decrease in cell count from control values p<0.05.

Fluorescence Microscopy Study

As MEMPL-treated U937 cells were stained with ethidium bromide and acridine orange in fluorescence microscopic analysis, it confirmed the presence of apoptotic cells (both early and late) when compared to untreated control cells. Various nuclear alterations were identified, including condensation of chromatin and the formation of apoptotic bodies. Both of this indicate the occurrence of apoptosis in U937 cells.
Figure 3: Fluorescence microscope images of U937 cells treated with IC₅₀ dose of MEMPL. The control cells give a bright green fluorescence whereas the MEMPL treated cells show an orange-red colour, demarking the occurrence of apoptosis in U937 cell.

Mitochondrial Membrane Potential Study

Another way for studying apoptosis is to disrupt the mitochondrial membrane potential. Because of the lipophilic nature of the JC-1 dye, the Mitochondrial Membrane Potential (m) was lost when leukemic cells were given the desired amount of MEMPL. It cannot remain in the mitochondria of an apoptotic cell due to mitochondrial membrane potential malfunction. After this, living cells produce red pigment (P3), while apoptotic cells emit green fluorescence (P4). When leukemic cells were treated with the IC₅₀ dose of MEMPL, the transmembrane shift increased.

Detection of Apoptosis by Flow Cytometry

In the flow cytometric analysis, double labelling technique, using annexin V FITC and propidium iodide, was utilized. Lower left (LL) quadrant (annexin V-/PI-) is considered 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+) signifies the cell population at late apoptotic stage and extreme upper right (UR) & upper left (UL) quadrant (annexin V-/PI+) is considered as necrotic cell population. Flow cytometric data analysis revealed that after 24 h of treatment with desired dose for quantification of apoptosis was observed for U937 1.9% against 46% were in lower right quadrant. Which signify the MEMPL have apoptotic inducing property on U937 cell line.

Cell Cycle Study

Flow cytometry also requires for the distinguishing of proof at different stages of the cell cycle. It also helps in the determination of relative cell DNA substance and provides information on cell position in the cell cycle. In U937 cells, flow cytometric analysis revealed large increases in DNA content in the G1 phase of the cell cycle, from 48.9 percent to 56.2 percent.

Figure 4: Showing Flow cytometric analysis of mitochondrial membrane potential (Δψm) on U937 cell line in untreated and MEMPL treated cells. A significant shift of membrane potential from red to green fluorescence was observed in U937 cells respectively.

Figure 5: Showing dual parameter of flow cytometric analysis of control and MEMPL treated U937 cell 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.
apoptotic cells, the mitochondrial membrane potential is disrupted, and the JC-1 dye is unable to enter within the mitochondria. The release of cytochrome C was induced by a transmembrane shift from red to green fluorescence caused by this depolarization. After staining with JC-1 dye, U937 cells treated with MEMPL showed a significant shift in transmembrane potential from red to green fluorescence. Healthy cells with predominately red fluorescence can be separated from apoptotic cells with principally green fluorescence. From the result, the findings suggest that a shift in transmembrane potential induces apoptosis. Another study compares the quantitative impact of early vs. late apoptosis in cells. It was also supported by dot plot assay which used dual staining with annexin V FITC and propidium iodide. Phosphatidylserine (PS) externalization is a hallmark of apoptosis, and various indicators for the study have been discovered. The binding of Annexin-V, which is labelled with FITC, is permitted by the translocation of PS molecules to the cell’s external surface. In dot plot assay, it was possible to identify live, early apoptotic, and late apoptotic cells using a dual staining technique with annexin V FITC and propidium iodide. The study showed the increase in the number of early and by the treatment with MEMPL of IC50 dose in U937 cells. One of the main causes of cancer progression is the deregulation of checkpoints. It eliminates cell irregularities and assures that cells follow the correct sequence of events of cells through the cell cycle’s different phases. From the study of cell cycle arrest by flow cytometry, it clearly proved that arresting cell populations in the sub-G0/G1 phases of the cell cycle slowed the growth of the U937 cell line.

CONCLUSION

The results of the present investigation that the methanolic extract MEMPL, isolated from Murraya paniculata leaves induced cell apoptosis and inhibited cell growth in human myeloid Leukemia (U937) cell line. MEMPL induced cytotoxicity involved DNA fragmentation, transmembrane shift, and cell cycle arrest. The cytotoxicity of the MEMPL is also confirmed by MTT assay. Microscopic images of MEMPL-treated cells revealed chromatin condensation and the formation of apoptotic bodies, indicating that apoptosis had occurred. Also, we identified that cell populations in the G0/G1 phase of the cell cycle had number of cells

DISCUSSION

Cancer is the largest cause of death worldwide, with approximately 10 million deaths predicted in 2020, accounting for nearly one in every six deaths. Lifestyle is responsible for up to 95% of all cancers, which can take 20–30 years to develop. Cancers of the blood cells are referred to as leukemia. The kind of leukemia is determined by the type of cancerous blood cell and its rate of growth. Many people with leukemia that grows slowly don’t show any signs or symptoms. The course of treatment varies widely. Treatment may include monitoring for slow-growing leukemia. Chemotherapy is followed by radiation and a stem-cell transplant and used to treat aggressive leukemia. Natural-derived substances are gaining popularity among scientists and specialists since they have less severe side effects than conventional treatments like chemotherapy. Murraya paniculata is an anti-inflammatory, antibacterial, and analgesic herb that has been used in traditional medicine for centuries. Therefore, we investigated the methanolic extract of Murraya paniculata leaf on against human myeloid Leukemia (U937) cell line. MTT assay results indicated antiproliferative and cytotoxic properties. The extract reduced cell growth and metabolic activities of the cell line in a time and dose dependent way. According to this study, MEMPL has an effect on leukemic cells. The effect of MEMPL on cell morphology was investigated further using a fluorescence microscope. Several morphological signs of apoptosis can be observed, including cell shrinkage, fragmentation into membrane-bound apoptotic cell, and rapid phagocytosis by nearby cells. After staining with acridine orange and ethidium bromide for fluorescence microscopy, the fluorescence microscopic images of the cells clearly showed nuclear degeneration of MEMPL treated U937 cells, compared to untreated control cells. The untreated control cells emitted bright green colour and had an intact cell membrane, as it prevents to enter ethidium bromide in case of fluorescence microscopy. MEMPL treatment causes apoptosis in the cells according to this research. Cell cycle phase distribution was also studied by flow cytometrically at IC50 doses in control and MEMPL-treated U937 cells. Mitochondria of the normal cells can take JC-1 dye and produce red pigment. But in

Figure 6: Flow cytometric evaluation of cell cycle phase distribution in control and treated MEMPL at IC50 dosage on U937 cells. Histograms represent increase in DNA content with actual number of cells which demonstrating the apoptosis inducing MEMPL on U937 cell line.
arrested. However, more research should be conducted to determine the active compound responsible for those activity in cells. As a result, there is a chance of developing a natural and novel anti-leukemic agent and it will lead us make it a potential treatment option for leukemia patients.

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Authors Contributions

S.B. contributed experiments, extraction, collection, and identification of specimen of Test Sample and Dr. S.E.B. contributed manuscript preparation, data curation and identification of specimen of Test Sample and Dr. S.E.B.

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