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Original Article

Evaluation of Cytotoxic Potential of Classical Siddha Medicine Padikara Parpam in Human Monocytic Leukemic Cell Lines (THP-1)

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ABSTRACT

Introduction: Padikara Parpam (PP), is one among the highly regarded Siddha medicine that is been traditionally claimed for its anti-proliferating and anti-carcinogenic properties. **Objectives:** In the present work, we investigated the cytotoxic effects of PP in a cellular model of Human leukemia (Human monocytic cell lines (THP-1). **Materials and Methods:** Determination of cell viability was assessed by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay and by using Confocal Laser Scanning Microscope (CLSM). Fluorescence-activated single cell sorting (FACS), and 2'-7'-Dichloro-Dihydro-Fluorescein Diacetate (DCFH-DA) mediated intracellular Reactive Oxygen Species (ROS) measurements were also carried out to understand the effect of PP on THP-1 cells. **Results:** PP induced cytotoxic effects against THP-1 in a concentration-dependent manner (6.02%–92.7%), with the highest cytotoxicity at 0.5 mg/mL concentration of PP. The IC_{50} values of PP in THP-1 cell lines were 0.115 mg/mL. The result from the MTT assay was further confirmed by CLSM reports. The images of Acridine orange/Ethidium bromide stained THP-1 cells treated with PP (IC_{50} concentration) indicated red fluorescence compared to the control cells which showed only green fluorescence. The images indicated the induction of apoptosis by the study drug. FACS and DCFH-DA based intracellular ROS measurements indicated the ability of PP to increase intracellular ROS levels in a concentration dependent manner. Thereby indicating at a possible ROS mediated apoptotic mechanism of action. **Conclusion:** These results suggest that with further clinical studies, PP could be used as an economic and effective anti-leukemic drug in patients suffering from leukemia.

Keywords: Siddha medicine, Padikara Parpam, Human Monocytic cell lines, Cytotoxic effects, Confocal Laser Scanning Microscope, MTT assay, Intracellular ROS assay.

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INTRODUCTION

Acute Monocytic Leukemia (AMOL) is one of the specific subtypes of Acute Myeloid Leukemia (AML) commonly occurring in young adult males, featured by clinical manifestations of bone marrow failure associated with bleeding, hemorrhagic gangrene over the extremities, hypertrophy of gingival region, ulceration, frequent infections, lymphadenopathy and hepato-splenomegaly.[1,2] Till now with various chemical therapeutic agents, the success rate is low, resulting in a high mortality rate among patients with AMOL.[3] There is a huge void in the field of cancer therapeutics which could be met with Indigenous Systems of Medicines (IMS). Effective time-tested drug candidates are the need of the hour and therefore the deciphered knowledge from each IMS has to be taken into consideration for improving their life expectancy and quality of life.

Siddha medicine, one of the leading Ayush Medical Systems has already proven its evidence-based approach in oncology.[4] The system follows a harmonized way of managing dreadful diseases like leukemia. Single plant therapy, herbal polytherapy, herbo-mineral therapy, and mineral therapy are actively graded based on the severity of the conditions.[5] drugs isolated or prepared from mineral resources offer magnus of opportunities in Siddha oncology and one such formulation called Padikara Parpam (PP) is focused in this cell line studies.

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PP is known for its exquisite effects in cancerous and non-cancerous tumors, and its supportive role in symptomatic control is well acknowledged from traditional experiences and literature credentials.[6] To strengthen the traditional claim over a scientific basis, here the study was purposed to identify the cytotoxic potential of PP on human monocytic cell lines.[7,8,9]

MATERIALS AND METHODS

Study drug

The sample for *in vitro* studies, PP was procured from a reputed GMP pharmaceutical, Tamil Nadu. The main ingredient of this calx formulation is alumen processed in egg white that was calcined to prepare a white micro-fine powder.

Chemicals and reagents for Cell line Assays

Phosphate Buffered Saline (PBS) and trypsin were procured from sigma aldrich co, st louis, USA. Acridine Orange (AO), Ethidium Bromide (EB), Glucose and antibiotics were purchased from Hi-Media Laboratories Ltd., Mumbai.

Preparation of Extract

A stock of 10 mg/ mL of PP was prepared in sterile PBS and diluted as needed for the effective final concentration.

Cell lines and Cell culture medium

Human monocytic cell lines (THP-1) were obtained from the National centre for Cell Sciences (NCS), Pune, India. Cells were grown in rpmi-1640 supplemented with 20% heat-inactivated Fetal Bovine Serum (FBS), and antibiotics like penicillin (100 IU/ mL), streptomycin (100 mg/mL), and amphotericin B (5 mg/mL) in a humidified atmosphere (5% CO_2) at 37°C until confluent.

Determination of Cell viability by MTT assay

The effect of PP on THP-1 cell viability was assessed using an MTT assay as previously described by Shazia Anjum *et al.* (2021) with minor modifications.[10] The compounds were tested at different concentrations. A stock of 10mg/mL was prepared for the sample and varying volume (1.25–20 µL) of the stock was added to the sterile tubes containing 20,000 cells/ 200 µL (as triplicates) and incubation continued for 24 hr. After incubation, the cells were pelleted and resuspended in 200 µL fresh media. MTT (5 mg/mL) was added to the cells and incubation continued for further 4 hr. Cells were then washed, pelleted, and incubated with Dimethyl Sulfoxide (DMSO). The DMSO solution was read at 570 nm using thermo multiskan go 96-well microplate reader. Cell viability was determined as the relative percentage of treated cells to the untreated (control) cells by using the formula,

Percentage (%) cell death = (Treated / Control) X 100

Determination of Cell viability using Confocal Laser Scanning Microscope (CLSM)

MTT results were confirmed by AO and EB staining method using CLSM. THP-1 cells were seeded, treated with the sample (0.115 mg/mL IC_{50}), and incubated as mentioned earlier. After 24 hr, the cells were pelleted and the supernatant was discarded. The cells without the sample were kept as control. These cells were then treated with PBS containing 50 µL of both AO–green fluorescence and EB–Red fluorescence and visualized under CLSM to image the dead cell population. All the cells will take up AO (green fluorescence), and only dead cells with compromised cell walls will take up EB (red fluorescence).[8]

Intracellular ROS Assay

Intracellular Reactive Oxygen Species (ROS) was measured using FACS / DCFH-DA in THP-1 cells by following earlier standardized method with minor modifications (Shazia Anjum *et al.* 2021).[10,11] The cells were cultured in a 6-well plate at a density of $1x10^5$cells/well. Cells were treated for 18hr with the compound (IC_{50} and IC_{25} concentration). Untreated and H_2O_2 treated cells were used as positive control. At the end of incubation, the cells were washed and resuspended 2% FBS in PBS and incubated with 10 µM of DCFH-DA for 30 min. After 30 min the cells where pelleted, washed and resuspended 2% FBS in PBS. Change in intracellular ROS levels was measured in correlation with change in fluorescence intensity using BD FACS calibre (Ex/Em–488 nm/530 nm). BD cell quest pro software was used to analyse the data.[10]

RESULTS

The cytotoxic effects of PP were evaluated on THP-1 cell lines using MTT assays. As shown in the Table 1 and Figure 1, there was marked cell death by PP in a concentration-dependent manner. The cells were exposed with PP for 24 hr, at the concentrations of 0.0313, 0.0625, 0.1250, 0.2500, and 0.5000mg/mL respectively. PP showed highest cytotoxicity (92.7%) at 0.5000 mg/mL concentration. The IC_{50} value of PP in THP-1 cell lines was 0.115 mg /mL. The percentage population death of THP-1 cells administered with different concentrations of PP is illustrated in Table 1 and Figure 1.

The CLSM images of THP-1 cells (control and test sample treated) stained with AO and EB is represented in Figure 2. In the observation, the THP-1 cells groups treated with IC_{50} concentration of PP showed significantly higher cells with red fluorescence compared to control cells. This indicates induction of apoptosis by the study drug. The CLSM results confirmed the earlier results from the MTT assay. Flow-cytometry based measurement of intracellular ROS levels indicated that there was increased intracellular ROS levels in THP-I cells due to the treatment of PP. (Figure 2) This increased ROS level was concentration dependent, with IC_{50} concentration of PP

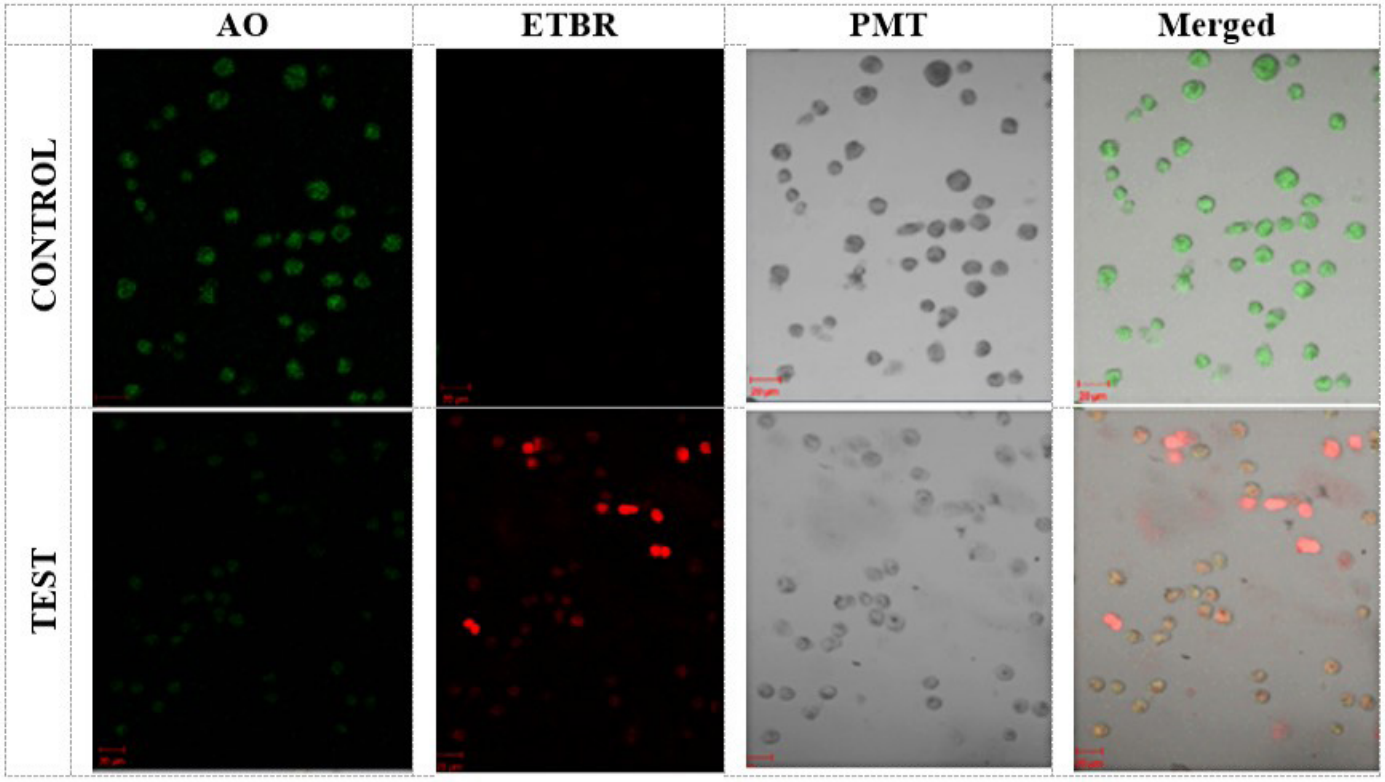


Figure 1: Confocal lazer scanning micrograph of THP-1 cells kept as control and Test samples stained with acridine orange (AO – green fluorescence) and ethidium bromide (red fluorescence).

AO = Acridine orange, ETBR = ethidium bromide, PMT = Photo multiplier Tube.

treatment showing increased fluorescence intensity compared to IC_{25} concentration of PP.

DISCUSSION

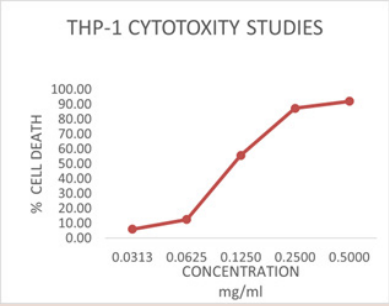
Most cancer therapeutic agents employ its effects in carcinogenesis through the central mechanism called apoptosis. However, varying degree of untoward effects is observed in most of the currently available agents.[12]

There is a need for attention towards potent natural drugs, and so far traditional references from Siddha medicine insist on the usage of selective mineral compounds which are used as target-specific anticancer agents. One among the frequently used inorganic salt mineral used in Siddha medicine is alum or alumen (potassium aluminium sulphate), which is commonly known in Tamil as padikaram.[6] This mineral in either raw or as prepared formulation (internal and external) is therapeutically indicated for its high-end possibility as a styptic agent in conditions like bleeding, discharge, and diarrhea al diseases.[6] It is specifically given internally at prescribed dosage forms for controlling heat excess in the body, inflammations, abnormal tissue growth, bleeding disorders like Idiopathic Thrombocytic Purpura (ITP), and leukemia.[12]

Alum by its astringent property can induce protein precipitation in the cell surface that further results in reduced capillary permeability. It is a vasoconstrictor and hardens the capillary endothelium leading to the control of inflammatory reactions like edema and exudation.[13] This makes it a suitable candidate for treating intravescicular hemorrhage due to bladder and prostate carcinoma.[14]

Table 1: Percentage of Cell Inhibition (THP-1 cells) treated with different IC_{50} concentrations of Padikara Parpam.

Concentration mg/mL	Cell death (in %)
0.0313	6.02
0.0625	12.57
0.1250	55.70
0.2500	87.31
0.5000	92.07



There are established studies of Alum proving its effects in tumor suppression by enhancing cell mediated responses. A study on Balb/c mice models induced with H22 Hepatocarcinoma followed by IP injections of alum had shown significant tumor growth reduction and increased cytokine profiles like IL-1β and TNF-α.[15,16]

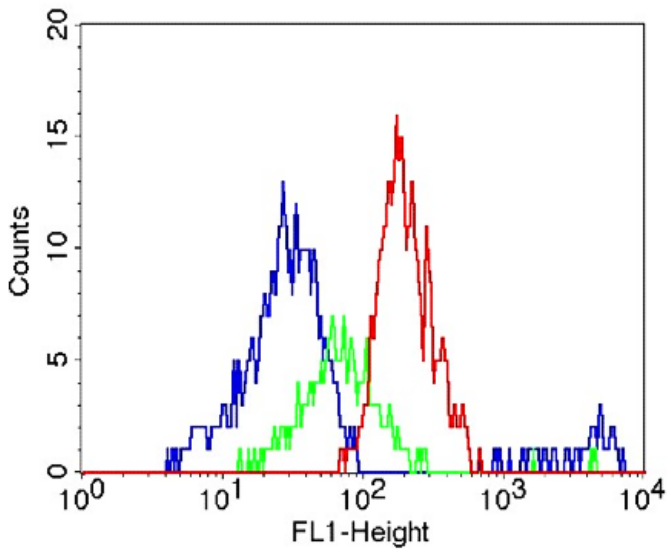


Figure 2: Intracellular ROS scavenging activity of Tested sample in THP1cells-Overlay histogram plots showing the concentration dependent increase in fluorescence signal due to increased intracellular ROS levels.

Blue–control, Green – lower concentration test sample, Red – Higher concentration test sample.

The calcined nano form of alum, PP is attributed to its specific usage in bleeding conditions associated with leukemia.[17] The mechanism of PP within the cancerous cells is still unclear, and in this perspective, this preliminary exploration was focused on the cytotoxic effects of PP against the human monocytic leukemia cell line (THP-1).

THP-1 has large and single-cell morphology and is considered a valuable cell line for exploring monocytes structure. Its utility in both health and disease is well appreciated in clinical oncological research.[18] The present study demonstrated the ability of pp to induce cell death in human monocyte leukemic cell lines. The results from the MTT assay clearly showed a direct correlation between the concentration of PP treatment and THP-1 cell death. The results were further confirmed by CLSM imaging experiments. The images of PP-treated THP-1 cells stained with AO and EB showed significantly high EB-stained cells compared to controls. This is once again a strong indication of study drugs ability to induce cell death in the leukemic THP-1 cells. Interestingly, the FACS based ROS assay indicates the ability of the study drug to increase intracellular ROS levels in THP-1 cells in a concentration dependent manner. Since increasing ROS levels are associated with cellular apoptosis, this is a possible mode of action of PP in THP-1 cells.[19]

The results while confirming the ability of traditionally used Siddha drug PP's ability to affect human leukemic monocyte cells, needs further investigation. There is a need to test PP on healthy human monocytes to study its biocompatibility. Experiments designed to assess the role of PP on the immunomodulatory system of the monocytes should also be done. Interestingly the Siddha drug PP is already a prescribed Siddha drug in use on human subjects suffering from leukemia. Thus, any finding about PP as a potential anticancer drug should facilitate a smoother transition of the drug into cancer therapeutics.

CONCLUSION

In the context of *in vitro* evaluation, the classical Siddha medicine Padikara Parpam had profound effects on acute monocytic leukemia cell lines. As the findings lead to a promising future approach for its management, more studies are warranted to establish its role in inducing apoptosis in cancer cells as well as its biocompatibility with healthy human monocytes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PP: Padikara Parpam; **CLSM:** Confocal Laser Scanning Microscope; **FACS:** Fluorescence-activated single cell sorting; **DCFH-DA:** 2'-7'-Dichlorodihydrofluoresceindiacetate; **ROS:** Reactive Oxygen Species; **AMOL:** Acute Monocytic Leukemia; **AML:** Acute Myeloid Leukemia; **IMS:** Indigenous systems of medicines; **PBS:** Phosphate Buffered Saline; **RPMI:** Roswell Park Memorial Institute culture; **DMSO:** Dimethyl Sulfoxide; **ITP:** Idiopathic Thrombocytic Purpura.

SUMMARY

With reference to the traditional claim in Siddha medicine of using Padikara Parpam in leukemic cases, here its cytotoxic effects were evaluated in THP-1 cell lines by using MTT assays, FACS and DCFH-DA mediated intracellular ROS measurements. The results from the MTT assay were further confirmed by CLSM reports. The medicines in a concentration-dependent manner were showing significant cytotoxicity and its ability to augment intracellular ROS levels. CLSM images indicated the induction of apoptosis by the study drug. The results proves the claim of its usage as an effective Anti-leukemic drug.

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