Research Article

Cell Growth Inhibition Study of *Murraya paniculata* (L.) Jack Leaves on Carcinoma Cells and Cell Cycle Arrest

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ABSTRACT

We evaluated the cell viability activity of methanolic extract of *Murraya paniculata* leaves (MEMPL) on hepatoma, gastric and colorectal carcinoma cell lines. In gastric (AGS) and both the hepatoma (HepG2 and Huh7) cells, the MEMPL significantly reduced the cell viability in a time and concentration dependent manner whereas in colorectal carcinoma (HCT116) the extract showed less significant reduction in comparison to hepatoma control cells. Morphological analysis revealed multiple markers of apoptosis in cancer cells, including chromatin condensation, nuclear fragmentation, formation of apoptotic bodies, and damaged DNA bands. Treated cells showed significant mitochondrial membrane potential shift in the mitochondrial transmembrane potential activity involving the release of cytochrome C into the cytosol. The cell cycle phase arrest was observed in the G0/G1 phase change in the MEMPL treated hepatoma cells. These findings indicate that MEMPL exhibited anticancer activity in cancer cell lines.

Keywords: *Murraya paniculata*, Leaf, Carcinoma, Apoptosis, Cell Cycle.

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INTRODUCTION

Cancer in Gastrointestinal tract (GIT) involves malignancy of the GI tract, and associated organs, which include the esophagus, stomach, bowel and rectum, liver, and pancreas.[1] Liver and colon cancer is the most common cause of death worldwide. Patients are frequently diagnosed with advanced liver cancer, which contributes to the disease's poor prognosis. The colon cancer is more common than the rectal cancer. There is a 2:1 or greater ratio of colon to rectum cases. With urbanization and industrialization, the incidence of these cancer rises. Colon cancer is unusual in people under the age of 45, with about 2 cases per 100,000 each year. Incidence of colon cancer is about 20 per 100,000/year in the 45–54 age group, and it rises at a significantly faster speed after that. Most of the GI cancer such as liver cancer and colorectal cancers are caused by advanced age and lifestyle choices, with a tiny number of instances caused by inherent genetic diseases. In this respect, 'oral chemotherapy' has provided a major leap forward when it comes to "Chemotherapy at Home".[2] The past decade has seen some major advances in the effectiveness of chemotherapeutic treatment for patients suffering from GI cancers. Due to the possible complications and adverse side-effects of surgical interventions in treating GI carcinoma, oral chemotherapy has gained much popularity with the prospect of providing bedside treatment for cancers. As a result, there is always a demand for new drugs to treat and prevent this life-threatening illness. Natural-derived substances are increasing in popularity among scientists and experts since they are regarded to have less harmful side effects than conventional treatments like chemotherapy. Anticancer effects of natural secondary metabolites produced by plants are being studied, which could lead to the novel therapeutic drugs. [3] New technologies are emerging to further improve the field because of the success of these natural compounds, which have been made into mainstream cancer treatments due to the anticancer activity. In folk medicine *Murraya paniculata* acts as an anti-inflammatory, antibiotic and analgesic. Essential oil from *Murraya paniculata* had a remarkable level of selective cytotoxicity against hepatoma cells (Hepa 1c1c7). [4] Methanolic extract of the whole plant contains a significant amount of flavonoids and has excellent anti-oxidant properties.[5] In albino mice, the bark extract of *M. paniculata* has a significant analgesic effect.[6] So, we evaluate the effect of methanolic extract of *Murraya paniculata* leaves (MEMPL) against hepatocellular carcinoma (HepG2 and Huh7), gastric adenocarcinoma (AGS) and colorectal carcinoma (HCT116) cell lines.

MATERIALS AND METHODS

Chemicals and Reagents

DMEM and RPMI1640 (Gibco), fetal bovine serum(FBS), Trypsin (Gibco, USA), Penicillin-streptomycin (Biowest, Germany), Gentamycin (Nicholas, India), ethylene diamine tetra acetic acid (EDTA), Agarose (Puregene), Proteinase k (SRL), RNAse, HEPES, L-glutamine, MTT [3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide)] (Sigma), DMSO (dimethylsulphoxide), Acridine orange, Ethidium bromide, Propidium iodide, JC1 (Sigma), Chloroform, Methanol, isoamyl alcohol, and all other chemicals and reagents were of analytical grade and procured locally.

Cell Culture

Gastric adenocarcinoma (AGS) cell line, colorectal carcinoma (HCT116) cell line, hepatocellular carcinoma (HepG2) cell line, hepatocyte-derived carcinoma (Huh7) cell line were obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. The HepG2, Huh7 and HCT116 cells were cultured in DMEM media whereas AGS cells were cultured in RPMI 1640 medium. All the cells were routinely maintained and supplemented with 10% heat inactivated fetal calf serum, 1% penicillin - streptomycin and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.[7] During the sub culturing of cells, the adherent property can be diminished by adding 1x trypsin solution in the cell. In all the experiments, untreated AGS, HCT116, HepG2 and Huh7 cells were termed as control group.

Collection and Identification of Test Sample (*Murraya paniculata)*

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, then grinded into powder (180 gm) and soaked in about 350ml petroleum ether in room temperature and was repeated 2 times. From that process, Petroleum ether extract was obtained. It was further dissolved in 250 ml Chloroform and was kept for 48 hrs. This step was repeated two times and the Chloroform extract was obtained.[8] Then, it was further dissolved in 250ml of methanol in room temperature for 7 days with occasional shaking. The mixture was then filtered by filter paper and the filtrate was then evaporated by Rotary Vacuum Evaporator and then lyophilized for 4 days to produce methanol free extract. The sticky methanolic extract was obtained finally. It was kept in airtight container and sealed with para film. Finally, the container is kept in 4° C and designated as methanolic extract of *Murraya paniculata* leaf extract (MEMPL) for the experiments.[9] The MEMPL extract was then dissolved in 100 ml water and then partitioned into two parts. One part was dissolved into 200 ml ethyl acetate to obtain ethyl acetate fraction and rest was dissolved in butanol to obtain butanol fraction. The fraction was termed as Ethyl acetate fraction of *Murraya paniculata* leaf (EFMPL) and n-butanol fraction of *Murraya paniculata leaf* (BFMPL). Stock solution was prepared (3mg/ml concentration) in phosphate buffer saline (PBS) from which desired doses were tested.

Phytochemical Investigation of *Murraya paniculata* Leaf Extract

Test for flavonoid

When a few drops of magnesium turnings and concentrated HCl were dropped into the extract, it turned in to pink, scarlet, crimson red, or sometimes green to blue in presence of flavonoid.

Test for Saponins

1 ml of alcoholic extract was diluted to 20 ml with distilled water and shaken for 15 minutes in a graduated cylinder. When one-centimeter layer of foam appeared, it indicates the presence of saponins.

Test for Alkaloid

A minimal portion of solvent-free chloroform was mixed with alcohol extracts separately, then filtered after adding a few drops of dilute HCl. Various alkaloidal reagents such as Mayer's reagent (cream precipitate), Dragendorff's reagent (orange, brown precipitate), Hager's reagent (yellow precipitate), and Wagener's reagent (reddish brown precipitate) were all used to evaluate the presence of alkaloids.

Test for Carbohydrate

A little amount (200mg) of alcoholic extract was dissolved separately in 5 mL distilled water and filtered. Millon's reagent was used to thoroughly evaluate the filtrate. Millon's test**:** 2-3 ml water extract was taken with very small amount drops of alpha-naphthol solution in alcohol. After this, solution was shaken and concentrated. H_2SO_4 was added from the test tube's sides carefully. At the junction of two liquids, a violet ring was formed in the presence of carbohydrate.

Test for volatile oil

The extract with alcoholic solution of Sudan III develops red colour in the presence of volatile oils.

Test for Phenolic compounds

In a little amount of alcoholic extract 3-4 drops of 0.1 percent v/v Ferric chloride were added to the filtered sample, the colour changed to brownish green or blue, indicating the presence of phenols.

Test for Phytosterols

The plant extract was treated with chloroform and filtered. 5-6 drops of sulphuric acid were treated with filtrate and shaken gently before letting to stand. The development of golden yellow colour indicates the presence of triterpenes (phytosterols).[10]

Cytotoxicity Study by MTT Assay

To evaluate the cytotoxicity activity of MEMPL on HepG2, Huh7, AGS and HCT116 cell line by performing the MTT

assay, all the cells ($1x10^5$) were seeded in 96- well sterile plates separately and were treated with different concentrations (5, 10, 25, 50, 100µg/ml) of MEMPL for 24, 48 and 72 hrs.[11] The cell cytotoxicity was also studied with the Ethyl acetate fraction (EFMPL) & n-Butanol fraction (BFMPL) treated in HepG2 and Huh7 cell lines with same concentration. All the cells were grown in humidified atmosphere containing 5% CO_2 in an incubator at 37°C for 24 hrs. The untreated cells were considered as control. After 24, 48 and 72 hrs. 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. The MTT assay is a colorimetric assay for assessing the metabolic activity of the cells or cell viability of NAD(P)H dependent cellular oxidoreductase enzymes and represents number of viable cells present. These enzymes can reduce the tetrazolium dye MTT, which is yellow in colour, to insoluble purple colored formazan.[12] The intensity of the colour was measured at 570 nm by micro-plate manager (Reader type: Model 680 XR Bio-Rad Laboratories Inc.). The IC_{50} values were determined for the different cells from the O.D value.

Fluorescence Microscopy Study

HepG2 and Huh7 cells ($1x10^6$) treated with IC_{50} of MEMPL for 24 hrs. were observed using a fluorescence microscope for morphological changes. The untreated control cells and the MEMPL treated cells were observed separately, washed with PBS, and then stained with acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) (1:1). The cells were then immediately mounted on slides and observed under a fluorescence microscope for the morphological determination of the cells undergoing apoptosis.[13]

Confocal Microscopy Study

HepG2 and Huh7 cells ($1x10^6$) were treated with IC_{50} of MEMPL for 24 hrs. After that the untreated control cells and MEMPL treated cells were harvested and washed with ice cold PBS. The cells were then stained with 10µg/ml of propidium iodide separately for 5 min. After mounting on slides, the cells were observed to see the differences in nuclear morphology between the untreated and the MEMPL treated HepG2 and Huh7 cells under confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystem, Heidelberg, Germany) installed with an inverted microscope [Leica DM-7RB]. Images for propidium iodide were acquired from argon/krypton laser and UV laser line using 570 nm long pass filter for propidium iodide and 450 nm band pass filter for UV images.[14]

Detection of Apoptosis by Agarose Gel Electrophoresis

HepG2 and Huh7 cells were treated with IC_{50} of MEMPL for 24 hrs. The cells were harvested and washed twice with PBS. The cells were resuspended in 500 µl of lysis buffer (50 mM Tris-HCl, pH -8.0, 10 mM EDTA, 0.5% SDS), 100 µg/ml of proteinase K was added, and incubation was done at 55 °C for 1 hr. 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 TE 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.[15]

Mitochondrial Membrane Potential Study

In a flow cytometer, HepG2 and Huh7 ($1x10^6$) cells were treated with MEMPL at the required dose and left untreated as a control for 24 hours to assess the mitochondrial membrane potential activity of the cells. The cells were washed in PBS, pelleted, then stained with the JC-1 dye. For 15 minutes, the sample was incubated at 37°C. Mitochondrial membrane potential changes were determined 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 (Becton Dickinson).[16]

Study of Cell Cycle Arrest by Flow Cytometry

HepG2 and Huh7 cells were treated with MEMPL (IC_{50} dose) for 18 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, stained with Propidium iodide, and stored in the dark for 15 minutes. 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).[17]

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:

% Cell inhibition= 100x (O.D of control – O.D of the treated)/ O.D of control

Where, O.D refers to Optical density

The percentage cell viability was calculated by the formula:

Viable cells (%) = (Total number of viable cells per ml/ Total number of cells per ml) x100.

RESULTS

Phytochemical Investigation of methanolic extract of *Murraya paniculata* leaves (MEMPL)

The methanolic extract of *Murraya paniculata* leaves (MEMPL) were subjected to preliminary phytochemical

study for detection of alkaloids, carbohydrates, glycosides, flavonoids, saponins and lipids/fat etc.

Table 1: Phytochemical Screening of Methanolic Extract of *Murraya paniculata* leaves

Phytochemical Test	Observation
Test for Flavonoid	-
Test for Saponin	+
Test for Alkaloid	+
Test for Carbohydrate	-
Test for Volatile Oil	+
Test for Phenolic compounds	+
Test for Phytosterol	-

The sign '+' indicates presence of the constituent and '-'indicates the absence of constituents.

Cytotoxicity Study and Cell Growth Inhibition Study

In the cytotoxicity study by MTT assay, there was significant reduction in the O.D values after treating the HepG2, Huh7, AGS and HCT116 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 10.83 µg/ml on HepG2 cell lines, 18.46 µg/ml on Huh7 cell line, 8.54 µg/ml on AGS cell line. But on HCT116 cells, IC_{50} dose of MEMPL was 83.11 µg/ml, which was less significant than other carcinoma cells. Further we have done cytotoxicity study on Ethyl Acetate fraction of MEMPL on HepG2 and Huh7 cells. The IC_{50} value of EFMPL calculated after MTT assay is 7.04 µg/ml and 18.46 µg/ml for HepG2 cells and Huh7 cells respectively.

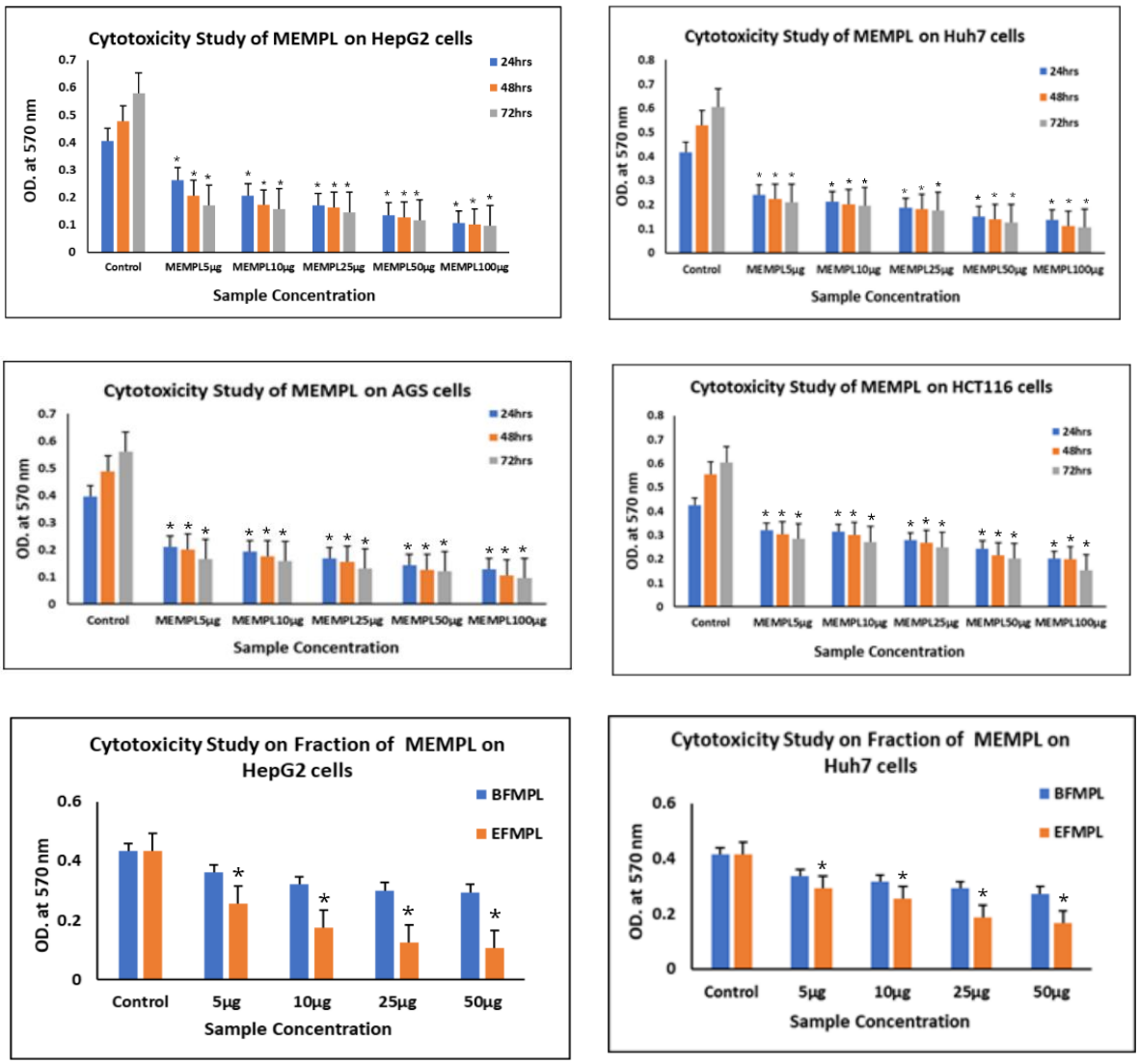


Figure 1: Histogram shows the cytotoxicity of MEMPL on HepG2, Huh7, AGS and HCT116 cells and in comparison, BFMPL fraction & EFMPL fraction on HepG2 & Huh7 cell lines after 24hrs. Reduction in the O.D at 570 nm is observed in a time and dose dependent manner. Data are mean ± S.E.M. * denotes significant decrease in cell count from control values $p<0.05$.

Fluorescence Microscopy Study

The observation of Fluorescence microscopic study on MEMPL treated HepG2 and Huh7 cells stained with ethidium bromide and acridine orange, revealed the presence of apoptotic cells (both early and late) as compared to the untreated control cells. Various nuclear alterations were identified, including chromatin condensation and the formation of apoptotic bodies. Both of this indicate the occurrence of apoptosis in HepG2 and Huh7 cells.

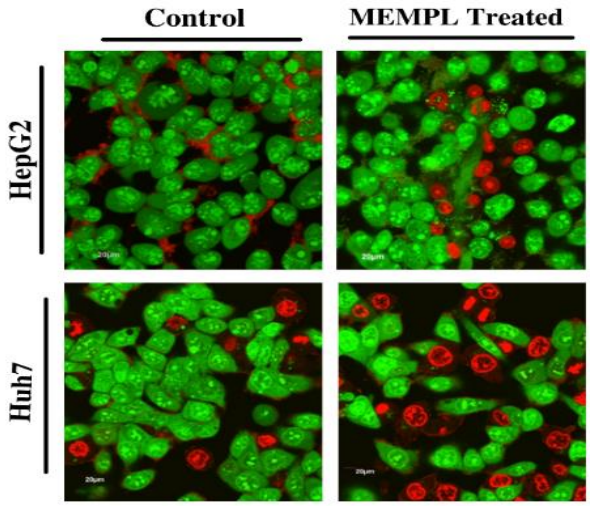


Figure 2: Fluorescence microscope images of HepG2 and Huh7 cells treated with IC_{50} dose of MEMPL. The control cells give a bright green fluorescence whereas the MEMPL treated cells show an orange-red colour, demarking the occurrence of apoptosis in HepG2 and Huh7 cells.

Confocal Microscopy Study

In comparison to untreated control cells, confocal microscopic observations of IC_{50} dose of MEMPL treated HepG2 and Huh7 cells stained with propidium iodide showed nuclear changes. Various nuclear fragmentations were found with the formation of apoptotic bodies and chromatin condensations . All of this indicate the occurrence of apoptosis in HepG2 and Huh7 cells.

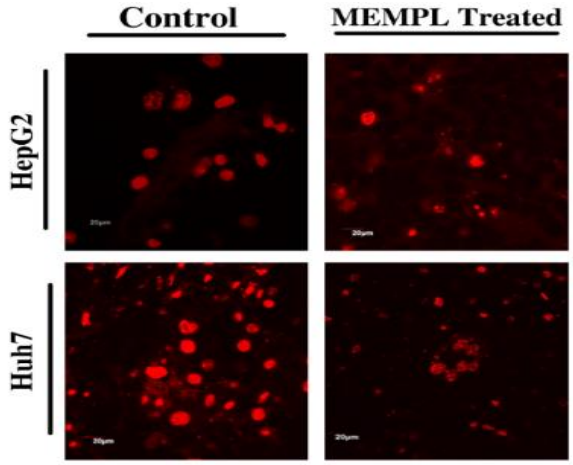


Figure 3: Confocal microscopic images of HepG2 and Huh7 cells treated with MEMPL showed apoptotic changes like nuclear disintegration and formation of apoptotic bodies stained with propidium iodide whereas untreated control cells have intact nucleus.

Detection of Apoptosis by Agarose Gel Electrophoresis

The gel pattern of the DNA samples isolated from untreated control HepG2 and Huh7 cells showed intact DNA bands whereas the gel pattern of the DNA samples isolated from MEMPL treated HepG2 and Huh7 cells showed degraded DNA bands in the form of ladders. So, the observations confirmed that the treatment with MEMPL caused apoptosis in both the carcinoma.

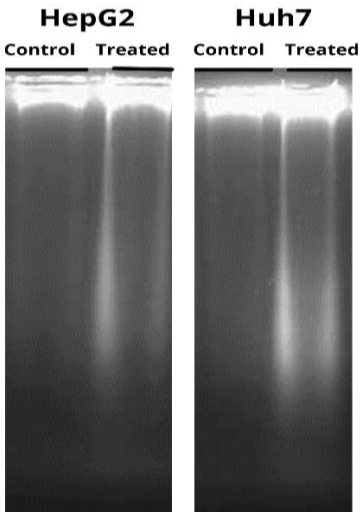
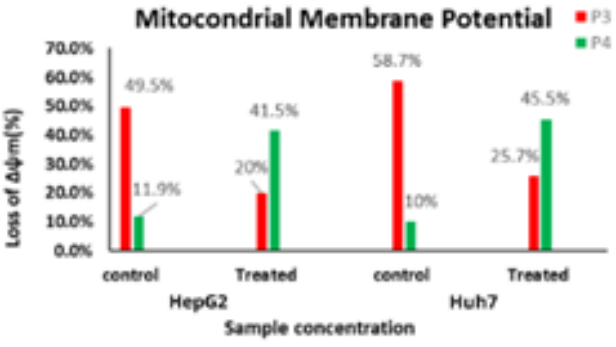


Figure 4: Showing agarose gel electrophoresis study of control and MEMPL treated HepG2 and Huh7 cell with IC_{50} dose. The control of HepG2 cell showed thick band of DNA whereas MEMPL treated cell showed laddering pattern in DNA.

Mitochondrial Membrane Potential Study

Disruption of mitochondrial membrane potential is another method for studying of the apoptosis. When gastric and hepatoma cells were given the desired amount of MEMPL, the Mitochondrial Membrane Potential (m) was lost, because of the lipophilic nature of the JC-1 dye. It cannot stay in the mitochondria of an apoptotic cell due to mitochondrial membrane potential malfunction. Furthermore, living cells produce red pigment (P3), while apoptotic cells emit green fluorescence (P4). When GI and hepatoma cells were treated with the IC_{50} dose of MEMPL, the transmembrane shift increased.



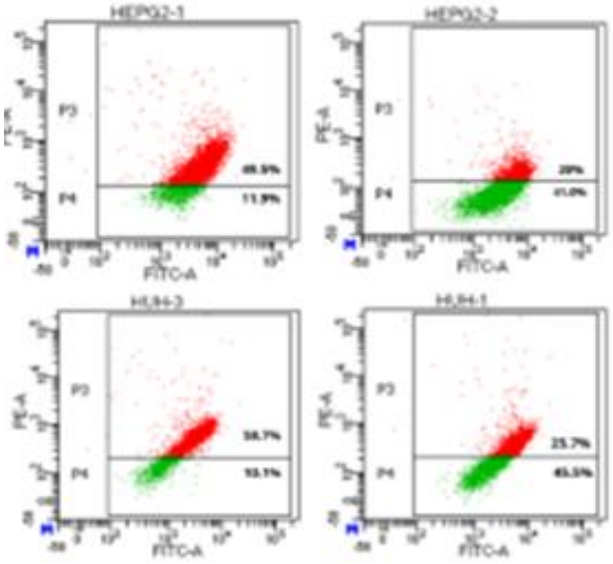


Figure 5: Showing Flow cytometric analysis of mitochondrial membrane potential (Δψm) on HepG2 and Huh7 cell line in both untreated and MEMPL treated cells. A significant shift of membrane potential from red to green fluorescence was observed in HepG2 and Huh7 cells respectively.

Cell Cycle Study

Flow cytometry also provides for the distinguishing of proof at different stages of the cell cycle. It also aids in determining the relative cell DNA substance and provides information on cell position in the cell cycle through this way. In HepG2 and Huh7 cells, flow cytometric analysis revealed large increases in DNA content in the G1 phase of the cell cycle, from 64.9 percent to 81.6 percent and 53.3 percent to 72.9 percent respectively.

DISCUSSION

The global burden of cancer is increasing, and it is now the second greatest cause of death. So, it catches the attention of the researcher and leads to the development of a potent drug. Because of adverse effects and drug resistance in cancer treatment, developing new drugs are still a difficult task. The use of natural methods aids us in overcoming such a challenge.[18] Natural products provide a valuable resource for effective drug development. *Murraya paniculata* plant is used to treat various diseases.[19] *Murraya paniculata* plant is used as an anti-inflammatory, antibacterial, and painkiller in traditional medicine.

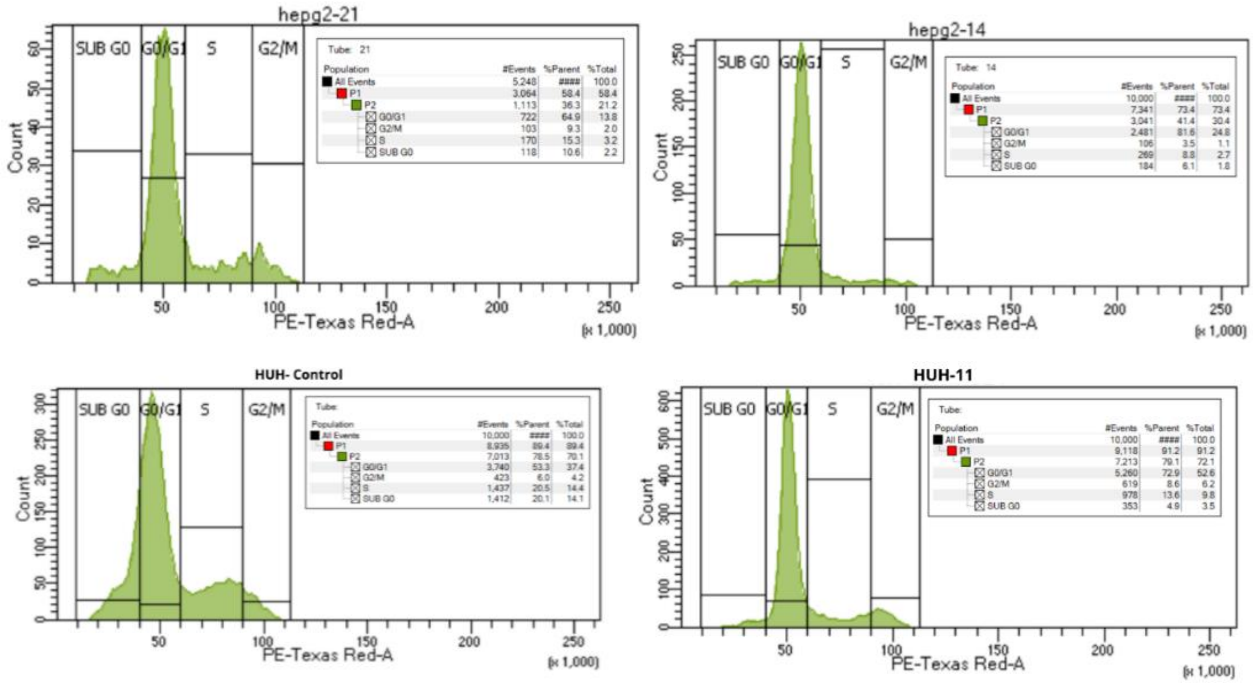


Figure 6: Flow cytometric evaluation of cell cycle phase distribution in control and treated MEMPL at IC_{50} dosage on HepG2 and Huh7 cells. Histograms represent increase in DNA content with actual number of cells which demonstrating the apoptosis inducing MEMPL on HepG2 and Huh7 cell line.

The methanolic extract of whole plants contains a significant number of flavonoids and has a great antioxidant property.[20] Therefore, we investigated the methanolic extract of *Murraya paniculata* leaf on gastric carcinoma, colorectal cancer and hepatocellular carcinoma cell lines. The antiproliferative and cytotoxic properties of MEMPL were confirmed by MTT assay results. The extract inhibited the cell growth and metabolic activities off the cell line in a time and concentration dependent way. This indicates that MEMPL affects gastric cancer, hepatocytic carcinoma and colorectal carcinoma cells. The effect of MEMPL on cell morphology was researched further using a fluorescent microscope and a confocal microscope. Cells were stained with PI and studied under confocal microscopy to check the chromosomal morphology. Membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation

and the formation of apoptotic bodies were the identified changes. As stained with acridine orange and ethidium bromide for fluorescence microscopy and propidium iodide for confocal microscopy, the fluorescence microscopic images of the cells clearly showed nuclear disintegration of MEMPL treated HepG2 and Huh7 cells, compared to untreated control cells. The untreated cells fluoresced bright green and had an intact membrane, preventing ethidium bromide from entering. On the other hand, MEMPL treated cells showed a mixture of orange-red fluorescence, because apoptotic and necrotic cells were unable to exclude the dyes and produced a blend of orange red. So, the research showed that treatment with MEMPL caused apoptosis in the cells. Furthermore, the anticancer activity of MEMPL was studied by the gel patterns of agarose gel electrophoresis of HepG2 and Huh7 cells treated with MEMPL. Untreated cells displayed one thick band of DNA, but MEMPL-treated cells showed fragmented DNA in a single lane. This simply means that the MEMPL can successfully degrade the genome of the cell lines. Cytometric evaluation of cell cycle phase distribution was also done at IC_{50} dosage in control and MEMPL treated HepG2 and Huh7 cells. JC-1 dye can enter the mitochondria of normal cells and develop red pigment. The mitochondrial membrane potential is disrupted in apoptotic cells, and JC-1 dye is unable to stay within the mitochondria. This depolarization caused a transmembrane shift from red to green fluorescence, resulting to the release of cytochrome C. HepG2 and Huh7 cells treated with MEMPL show a significant shift in transmembrane potential from red to green fluorescence when stained with JC-1 dye. Apoptotic cells with principally green fluorescence can be differentiated from healthy cells with predominantly red fluorescence. Hence, the results point to a change in transmembrane potential triggering apoptosis. From the study of cell cycle arrest by flow cytometry, it clearly demonstrated that arresting cell populations in the sub-G0/G1 phases of the cell cycle slowed the growth of the HepG2 and Huh7 cell line.

CONCLUSION

From this investigation, we found that the methanolic extract MEMPL, isolated from *Murraya paniculata* leaves induced cell apoptosis and inhibited cell growth in gastric, hepatoma and colon cancer cell lines. Further microscopic images of MEMPL-treated cells revealed membrane blebbing, cell shrinkage, chromatin condensation, and the development of apoptotic bodies, indicating that apoptosis had occurred. The cytotoxicity of the MEMPL is also indicated by MTT assay. DNA fragmentation of these cells are confirmed through agarose gel electrophoresis. Also, we observed that the number of cells arrested with cell populations in the G0/G1 phase of the cell cycle. So, the results confirmed that plant can be used to develop as a potential anticancer agent. It will lead us in making it a promising therapeutic option for people with gastric, colon and liver cancer.

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AUTHORS CONTRIBUTIONS

Shreya Biswas contributed experiments, extraction, collection, and identification of specimen of Test Sample and Dr. Shila Elizabeth Besra contributed manuscript preparation, data curation and manuscript improvement.

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