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Original Article

Evaluation of the cytotoxic activity of leaf extracts of *Mimosa rubicaulis* (Lam.) against cancer (HepG2) and normal (L929) cells through induction of apoptosis

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Abstract

Investigation of the *in-vitro* anti-cancer and apoptosis activity of various extracts of *M. rubicaulis* (Lam.) against cancer cell lines (HepG2 and L929) is important. The HepG2 and L929 Cell lines were exposed to increasing concentrations of various extracts of leaves of *M. rubicaulis* (Lam.) ranging from 640 to 20 µg/ml for 24 hours. MTT assay was used to determine cytotoxicity. All extracts of leaves of *M. rubicaulis* (Lam.) treated with cancer cell lines HepG2 and normal cell line L929 in response to increasing concentration, cell viability decreased significantly. Additionally, the most affected cells were HepG2 cells, followed by L929. The study found that when exposed to ethanolic extract, the cancer cell lines (HepG2) showed the highest expression, while the normal cell lines (L929) showed the lowest. Based on the experimental data, we discovered that *M. rubicaulis* (Lam.) has a cytotoxic effect on cancer cell lines. In contrast, no cytotoxic effect was observed at the highest dose on normal cells. The ethanolic extract had potent anti-cancer activities against HepG2 cells *via* induction of Apoptosis by Flow Cytometry. According to the findings ethanolic extract has a high cytotoxicity against HepG2 cells, with an IC_{50} of 93.69 µg/ml. Apoptosis processes such as alterations in cell shape, chromatin condensation, membrane swelling, and the production of apoptotic bodies were seen in ethanolic extract treated HepG2 cells. The ethanolic extract treated HepG2 cells variations in light scattering reveal the general characteristics of cell death due to apoptosis. These outcomes show how effective ethanolic extract of *M. rubicaulis* (Lam.) to exert apoptosis, particularly in late stage apoptosis in HepG2 cell lines. Hence, further investigation is required to study the phytoconstituents in ethanolic extract of *M. rubicaulis* (Lam.) responsible for cytotoxic activity.

Keywords: Cytotoxicity, *M. rubicaulis* (Lam.), Anti-cancer potential, Apoptosis, HepG2, L929, MTT assay, IC_{50}.

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1. Introduction

Aggressive cancers are the second most significant cause of death among people [1]. Hepatocellular carcinoma/(HCC) is one of the riskiest types of cancer, with a bad prognosis,

high rates of morbidity, and death [2]. HCC is becoming more prevalent in China, where it accounts for 90% of all primary liver cancer cases, making it the second most significant cause of death [3]. However, there are few treatment options for HCC [4-6]. Currently, the most popular treatment for cancers of the liver and other organs is chemotherapy. On the other hand, a significant obstacle to the effective use of chemotherapy in the treatment of cancer is the toxicity of chemotherapeutic drugs in healthy tissues and cells. As a result, there is a pressing need to discover new hepatoma therapeutic agents. Plants contain secondary metabolites and phytochemical substances that may be therapeutic. Derivatives of naturally occurring compounds account for nearly 60% of all current drug treatments in use. When compared to commercially manufactured chemotherapeutic agents, because of their availability, low cost, and lesser side effects, medicinal plants are increasingly being used to treat cancer. Because there is a need for efficient therapeutic approaches to treat diseases like cancer, medicinal plants are a strong choice because of their low toxicity profile in normal cells while fighting various types of cancer [7]. The cell's inherent process for planned cell death is called apoptosis. It is a highly regulated process that removes any undesirable or superfluous cells. External and intracellular cues can trigger the apoptotic process. The intrinsic and extrinsic processes correlated with the signal type are the two distinct pathways that lead to apoptosis [8]. Anti-cancer drug cytotoxicity relies heavily on apoptosis, or programmed cell death. A series of gene-regulatory cell-signaling pathways aid the induction of apoptosis; unique biochemical and morphological alterations accompany this. The apoptotic cascade has previously been shown to require disruption of mitochondrial function. Anticancer drugs may cause mitochondrial damage by increasing the permeability of the outer mitochondrial membrane, which is associated with the breakdown of the mitochondrial membrane potential, as a decrease in inner mitochondrial membrane potential($\Delta\Psi m$) disrupts intracellular ATP synthesis, reactive oxygen species production, the mitochondrial redox ratio, cytochrome C translocation to the cytosol, and caspase-3/poly ADP ribose polymerase degradation.

Given that alterations occur in the mitochondria early in the apoptotic process, it is thought that the mitochondria are necessary for triggering of apoptosis [9-14]. Previous research has shown that the inner mitochondrial membrane potential ($\Delta\Psi m$) is involved in a cell's apoptosis regulation. When a cell undergoes apoptosis in response to a specific physiological signal, a proteolytic cascade involving several caspases is triggered, which causes nucleases to become active, resulting in the degradation of chromosomal DNA. The apoptotic process is characterized by DNA destruction of this type.

Caspases, a class of proteases, are activated in cells undergoing apoptosis. As a result, various molecular and structural changes occur, including nuclear heterochromatin condensation, DNA repair enzyme degradation, cell shrinkage, and loss of the cytoplasmic organelle positional arrangement [15-16]. Because there are few treatment options for

HCC, Researchers used the HepG2 human HCC cell line to examine the anticancer potential of oleanolic acid, a plant triterpene. It is the method of choice for apoptosis quantification, a procedure that uses DNA staining to distinguish between apoptotic and non-apoptotic cells. This multiparametric approach makes it possible to count, inspect, and categorize small particles suspended in a fluid stream. Electronic detection equipment that records forward scatter and side scatter can be used to analyze cells' physical and chemical features. This characteristic distinguishes Apoptotic cells from other cells [17-19]. No remarkable research has been done on *M. rubicaulis* (Lam.) leaf apoptosis by flow cytometry or cytotoxic activities by the MTT assay method employing HepG2 cell lines.

2. Materials and Methods

2.1. Collection, Authentification & extraction

2.1.1. Plant Material Collection, Authentification

Leaves of *M. rubicaulis* (Lam.) were collected from Dehradun. The leaves were carefully washed 2-3 times under running water and then distilled. After being sterile the components of the plant were then in the shade, dried and densely pulverized separately and stored in hermetically sealed bottles for further laboratory analysis and Government of India, National Institute for Traditional Medicine (ICMR-NITM) with access number RMRC-1446, and the Botanical Survey of India/BSI, Pune, Maharashtra, also verified the authenticity of *M. rubicaulis* (Lam.), "BSI, Northern- Regional Centre, 192, Kaulagarh-Road, Dehradun."

2.1.2. Preparation of Extracts

The leaves of *M. rubicaulis* (Lam.) remained shade-dried at 26 $\pm 2^0$C, the dried plant material (500 g) each was finely powdered and passed through a 0.3mm mesh-sized sieve. The solvent extraction was then done in stages using the hot percolation technique (Soxhlet extraction) with several solvents of increased polarity, starting with extremely non-polar solvents such as PE (60-80°C), $CHCl_3$, EA, and then ETH (95%), The cold maceration was used to prepare AQ extra. The extracts were stored in the refrigerator until they could be utilized again. All of the extracts were labeled correctly.

2.1.3. Preparation of standard solutions

Standard (Std.) drug was prepared by liquefying 100 mg of 5-fluorouracil in 20µl of DMSO and diluted to 10ml with PBS to give a 10000 µg/ml concentration. Of these, 640, 160 & 40 µl were taken out and adjusted to 10ml with PBS. The final concentrations are 640, 160 and 40µg/ml.

2.2 Growth media, conditions for treatments, cell culture, and maintenance of cell lines

HepG2 (Liver Cell Line) and L-929 (Normal Cell Line) were procured from the National Centre for Cell Science (NCCS). Cell lines were maintained in Dulbecco's modified eagle medium (DMEM) with fetal bovine serum (FBS) (10%), at 37ºC with 5% CO_2. Cell culture was performed using standard procedures in a laminar airflow chamber (Bergmann and Feeney, 1951). Antibiotic: Anti-mycotic 100X solution (Thermo Fisher Scientific) - Cat No-15240062.FBS (Gibco, Invitrogen) Cat No -10270106 [20].

2.3. Cell viability determination by MTT assay

For evaluation of cytotoxicity on different leaf extracts, we determined the IC_{50} value of extracts. The cell line was cultivated in DMEM media supplemented with 1% Antibiotic – Anti-mycotic 100 X solution and 10% heat-inactivated FBS. Seeding the cells at a density of approximately 5×10^3cells/well in a 96-well flat-bottom micro plate and maintained at 37°C in 95% humidity and 5% CO_2 overnight. Different concentrations (640, 320,160,80,40,20 µg/ml) of samples were prepared. One more day was spent incubating the cells. After twice washing the cells in the well with phosphate buffer solution, 20 µl of the MTT staining solution (5 mg/ml in phosphate buffer solution) was added to each well, and the plate was incubated at 37ºC. After 4 hours, 100 µl di-methyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals and the absorbance was measured using a 570 nm micro-plate reader [21].

2.4. Cell culture, maintenance of cell lines and growth medium for apoptosis assay

Cell lines–HELA (Cervical cancer) Dulbecco`s Modified Eagle Media (DMEM) with low glucose -Cat No-11965-092 (Gibco, In-vitrogen), Fetal-bovine serum (FBS) - Cat No - 10270106 (Gibco, Invitrogen), Antibiotic /Antimycotic 100X solution (Thermofisher Scientific)-Cat No-15240062, TACS Annexin V-FITC Apoptosis Detection Kit (R&D Systems) Cat No –4830-01-K.

2.5. Apoptosis Assay by Flow Cytometer

After being planted in a 24-well flat-bottom microplate with cover slips, the cells were kept in a CO_2 incubator overnight at 37ºC. Half of the maximum inhibitory concentrations (IC50) of ethanol extract (ETHE) were applied to the cells for 12 hours. Following incubation, PBS was used to wash the cells. Centrifuge at 500 x g for 5 minutes at 4ºC. The supernatant was discarded, and the cell pellets were resuspended in ice-cold 1X Binding Buffer to 1 x 105 per ml. Ice was used to store the tubes. After that, carefully mix in 1 µl of Annexin V-FITC solution and 5(five) µl of PI. Placed the tubes on ice and let them sit in the dark for 15 minutes. 400 µl of ice-cold 1X binding buffer was added and gently mixed. A flow cytometer was used to analyze the cell preparations in less than 30 minutes [22].

3. Results and Discussion

3.1 Cell viability determination by MTT assay of leaves of M. rubicaulis (Lam.)

Drug 5-FU, ETHE of *M. rubicaulis* (Lam.) leaves exhibits substantial cytotoxicity in the cell viability research on the HepG2 cell line compared to Std. With a decrease in cell viability percentage, the cytotoxic effect increases with concentration; i.e, at 640 µg/ml, there is a (28.35 ±1.03) less toxic effect, and at 20 µg/ml, there is a (62.56 ±0.31** %) more toxic effect.

Comparative IC_{50} values for HepG2 cells were displayed for petroleum ether extract (PEE), chloroform extract (CHCl3E), ethyl acetate extract (EAE), ETHE, and aqueous extract (AQE) in (Table 1). The most effective anti- proliferation effect was induced by ETHE with an IC_{50} value of 93.69 µg/ml, followed by EAE with an IC_{50} value of 190.56 µg/ml. AQE and $CHCl_3E$ showed a similar inhibitory effect

on cell growth with IC_{50} values of 492.74 and 502.70 µg/ml, Followed by PEE with IC_{50} values of 812.40 µg/ml respectively **(Fig. 1)**.

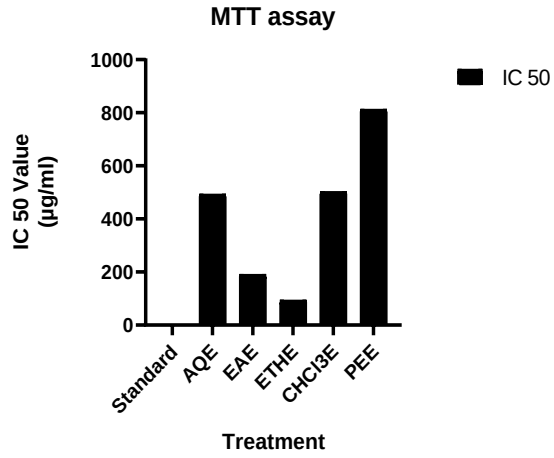

Figure 1. The IC_{50} value was obtained using the MTT assay, and the graph shows the IC_{50} values of different *M. rubicaulis* (Lam.) leaf extracts.

In **Figure 2** prior to treatment, normal cell line L929 was seen to consist of fibroblast cells that were spindle-shaped, flat, and elongated (aligned in a parallel cluster). The administration of various *M. rubicaulis* (Lam.) extracts at varying concentrations did not cause the cells to undergo any morphological alterations. The rate of cell division and the growth of untreated L929 cells were adequate. [24].

Figure 3 shows the morphological changes in HepG2 cells upon treatment with different *M. rubicaulis* (Lam.) extract concentrations. The ETHE of *M. rubicaulis* (Lam.) was the only extract that caused a significant change in the morphological characteristics of the tested cell lines. Cells shrank and lost their capacity to adhere to the surface of the culturing plate. Furthermore, cells seemed rounded and floated at the greatest applied dose compared to the control morphology [23].

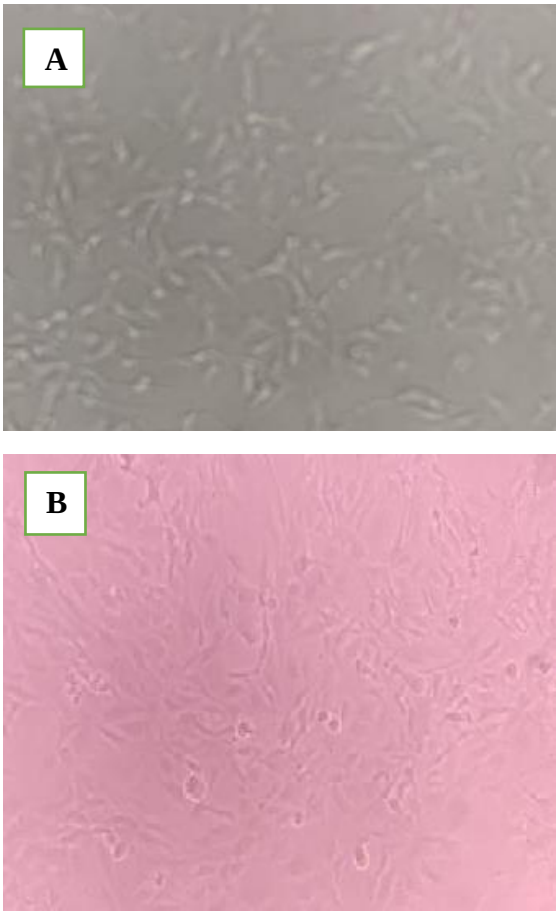

Figure 2. Normal Cell line before treatment and after treatment. A) L929 Normal Cell line before treatment, B) L929 Normal Cell line after high concentration

Flow-cytometric examination of (A) control and (B) *M. rubicaulis* (Lam.) of ETHE and Annexin V-FITC and PI double labelling were utilized. Both panels' results show (Annexin+/PI-) early apoptotic in the lower/downward right quadrant/(Q3).

Whereas (Annexin +/PI+) higher right apoptotic, i.e. late apoptotic quadrant/ (Q3) (Q2). The viable cells are shown by the quadrant on the lower left (Q4). Necrosis (Annexin -/PI+) is shown in the top left quadrant (Q1).

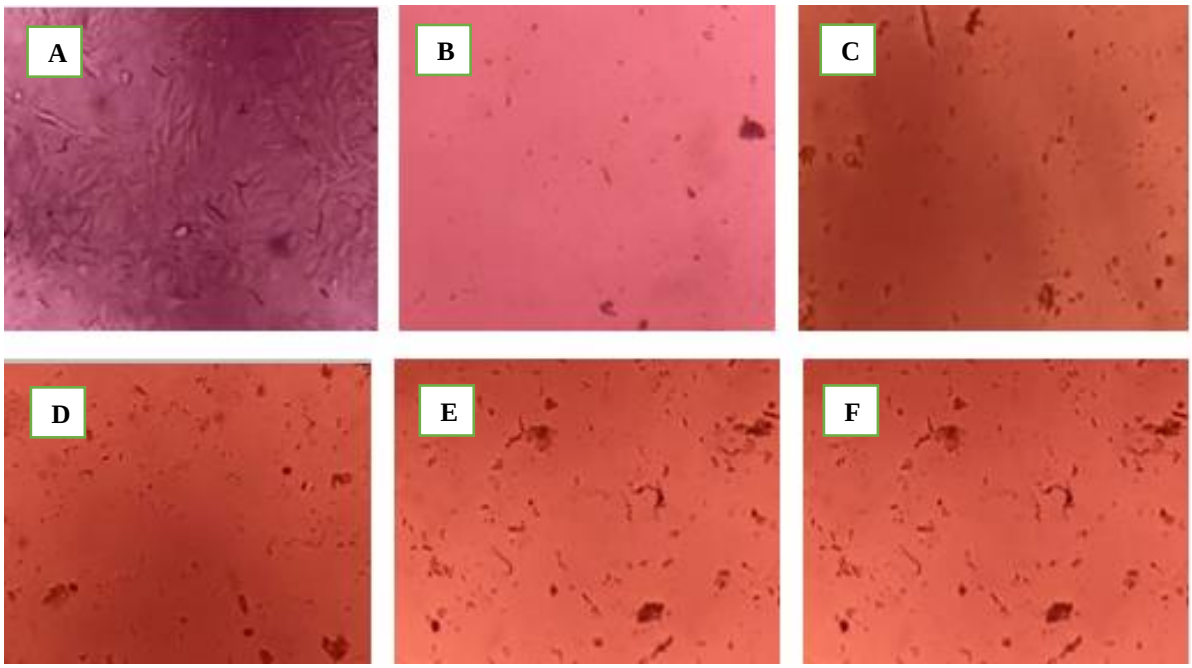

Figure 3. Morphological changes in HepG2 Cell Lines as affected by different extracts of *M. rubicaulis* (Lam.) after 24h. A) HepG2 before Treatment, B) HepG2 after treatment with AQE of *M. rubicaulis*(Lam.), C) HepG2 after treatment with EAE extract of *M. rubicaulis* (Lam.), D) HepG2 after treatment with ETHE of *M. rubicaulis* (Lam.), E) HepG2 after treatment with $CHCL_3E$ extract of *M. rubicaulis* (Lam), F) HepG2 after treatment with PEE of *M. rubicaulis* (Lam.)

Data is presented in **Table 1.** shows different extracts of *M. rubicaulis* (Lam.) Using the MTT assay, dramatically decreased the cell viability over a 24-hour incubation period in a concentration-dependent manner. ETHE of *M. rubicaulis* (Lam.) was more effective against HepG2 cell lines than the other extracts, namely PEE, $CHCl_3E$, EAE and AQE when the cells were treated with ETHE for 24 h. The IC_{50} value of ETHE in HepG2 cells incubated for 24 h was 93.69µg/ml. Lactate dehydrogenase (LDH) was also released from the cells when damaged. These results imply that ETHE can successfully inhibit the growth of HepG2 cells. 5-FU (IC_{50}=1.66 µg/ml after 24 hours) was employed as a standard or positive control. When compared to other treatments, 5-FU exhibited the lowest survival rate and was harmful at cell levels in a dose-dependent manner.

3.2 M. rubicaulis (Lam.) of ETHE-induced Apoptosis in HepG2

Four populations of cells were distinguished from our current results by Annexin-V/PI staining; Live cells (Q-4), early apoptotic cells (Q-3), late apoptotic cells (Q-2), and dead cells (Q-1) (**Figure 4**). Cell death was determined in the HepG2 cell line using FITC-conjugated Annexin-V membrane staining and PI nuclear counterstaining. Cell populations were quantified by flow cytometry analysis after 12 hours of treatment with half maximal inhibitory concentration (IC_{50}) concentrations of ETHE.

The treatment of HepG2 cell lines with *M. rubicualis* (Lam.) of ETHE resulted in a time-dependent change in the cell population from Live to Apoptotic, as demonstrated by flow cytometry analysis.

Table 1: Results of the MTT assay of different extracts of *M. rubicaulis* (Lam.) on Liver Cell Line-HepG2**.**

Treatment	Con. (µg/ml)	Cell Viability%	IC_{50} Value(µg/ml)
NC	-	100 %	-
Standard	640	10.58±1.17*	1.66
	160	20.25±1.23**	
	20	28.87±1.28***	
AQE	640	46.33 ±1.07	492.74
	320	53.91 ±1.33	
	160	59.26 ±1.56	
	80	66.46 ±1.39	
	40	69.31 ±2.25	
	20	73.28 ±1.41	
EAE	640	30.21±2.23	190.56
	320	45.02±1.91	
	160	54.87±0.50	
	80	62.70±1.19	
	40	70.07±1.89*	
	20	81.25±1.58*	
ETHE	640	28.35 ±1.03	93.69
	320	39.45 ±1.32	
	160	46.25 ±2.06	
	80	53.58 ±1.81	
	40	58.65 ±0.83**	
	20	62.56 ±0.31**	
$CHCl_3E$	640	48.30±1.91	502.70
	320	52.66±1.92	
	160	59.01±1.96	
	80	64.14±1.56	
	40	68.83±1.29	
	20	73.49±0.47	
PEE	640	50.91±1.82	812.40
	320	55.20±1.91	
	160	73.00±2.02	
	80	78.54±0.97	
	40	79.18±1.40	
	20	85.33±0.89	

Con. Concentration, Values are expressed as (Mean±SEM)(n=3); Data was analyzed -using one–way ANOVA by using Graph Pad Prism 9 for Windows. Disease Control Vs. Treated Groups, where (*p<0.05,**p<0.01, ***p<0.001).

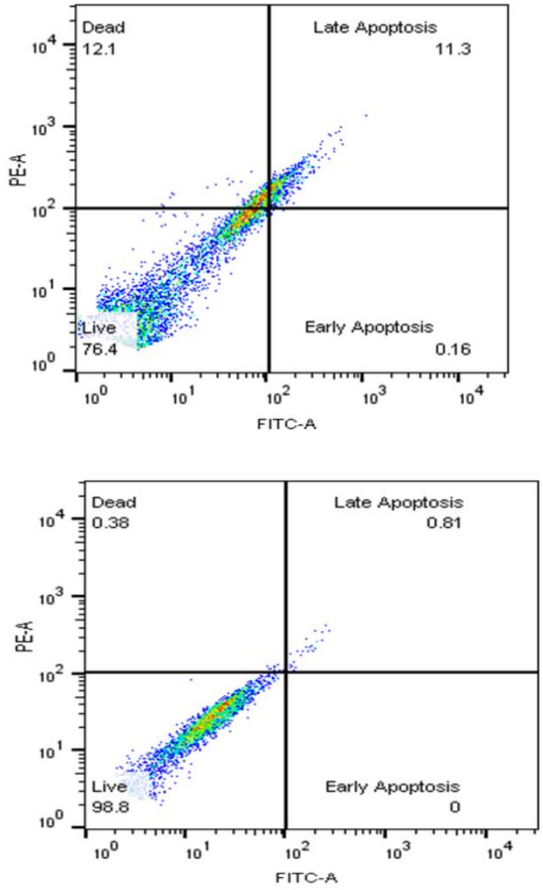

Figure 4: Results of Apoptosis on *M. rubicaulis* (Lam.) of ETHE and controlled

This shift occurred after 12 hours. When the early apoptotic rates were compared to the positive control rate (0.00%), they showed non-significant differences. Additionally, at 12 hours of incubation, there were significantly greater alterations ($p \leq 0.001$**) in the percentage of late-stage apoptotic cells for *M. rubicualis* (Lam.) of ETHE-treated HepG2 cell lines (11.3%), respectively, compared to (0.81%) of the positive control group. The dead cells of *M. rubicualis* (Lam.) of ETHE treated HepG2 cell lines were observed at 12 hours of incubation (12.1%), respectively as compared to (0.38%) of the positive control group. These

results demonstrated the ability of *M. rubicualis* (Lam.) of ETHE to exert an apoptotic effect on HepG2 cell lines particularly in the late stage of apoptosis which is an irreversible process the percentage of cells entered in the apoptosis phase was 11.3% for *M. rubicualis* (Lam.) of ETHE treated HepG2 cell lines **(Table 2).**

Table 2: *M. rubicaulis* (Lam.) of ETHE induced apoptosis in HepG2 cells**.**

Sr.No	Q-4	Q-3	Q-2	Q-1
Sample Code	**Live %**	**Early Apoptosis %**	**Late Apoptosis %**	**Dead %**
Control	98.8	0.00	0.81	0.38
***M. rubicaulis*(Lam)of ETHE**	76.4	0.16	11.3	12.1

The study aimed to see how *M. rubicualis* (Lam.) affects cell viability and apoptosis in HepG2 cells to figure out how it works as an anticancer agent. The cytotoxic effect of *M. rubicualis* (Lam.) extracts was assessed using the micro-culture tetrazolium assay (MTT) on some cell lines. IC_{50} values were computed using *M. rubicualis* (Lam.) extracts at various concentrations. The results of the cytotoxicity evaluation against cancer (HepG2) and normal (L929) cells lines are shown in (**Tables 1** and**3)**. *M. rubicualis* (Lam.) extracts exhibited no cytotoxic effect on the L929 cell line, with an IC_{50} value of more than 1000 µg/ml suggesting they are biocompatible across various concentrations.

Conversely, *M. rubicualis* (Lam.) extracts exhibited cytotoxic effects that were selective for HepG2 cell lines, with an IC_{50} value of less than 1000 µg/ml [25]. One characteristic that sets cancer cells apart is their resistance to apoptosis. [26-27]. Therefore, an in-depth understanding of the relevant apoptotic signaling pathways is essential for identifying and creating target-specific treatments. A valuable tool for researching carcinogens is the mouse model.

Table 3: Results of the MTT assay of different extracts of *M. rubicaulis* (Lam.) on Liver Cell Line-HepG2**.**

Treatment	Con. (µg/ml)	% Cell Viability	IC_{50} Value(µg/ml)
NC	-	100 %	-
Standard	640	10.58±1.17*	1.66
	160	20.25±1.23**	
	20	28.87±1.28***	
AQE	640	62.14±1.53**	3294.46
	320	64.78±2.57*	
	160	71.80±1.39*	
	80	77.60±1.80*	
	40	82.85±1.50*	
	20	84.60±0.83*	
EAE	640	56.9±1.41*	2697.28
	320	59.68±1.88*	
	160	62.61±2.37*	
	80	64.72±1.80*	
	40	68.69±2.88*	
	20	72.86±2.20*	
ETHE	640	59.34±1.60*	2591.52
	320	63.18±1.37*	
	160	64.24±1.73*	
	80	70.74±2.08*	
	40	75.45±1.44*	
	20	79.8±0.91*	
$CHCl_3E$	640	60.86 ±1.86*	2951.29
	320	64.84±2.50*	
	160	71.56±2.29*	
	80	77.84±1.49*	
	40	81.61±1.84*	
	20	84.54±3.03*	
PEE	640	62.51±1.80*	3640.95
	320	65.26±2.73*	
	160	70.68±2.41*	
	80	76.79±3.20*	
	40	82.9±0.26*	
	20	83.67±3.69*	

Conc. Concentration, Values are expressed as (Mean±SEM)(n=3); Data was analyzed -using a one–way ANOVA using Graph Pad Prism 9 for Windows. Disease Control Vs. Treated Groups, where ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

They will significantly contribute to our understanding of the cancer's molecular processes and causes. [28-29]. Therefore, the current study aims are to clarify the mechanism of cell death and the molecular mechanisms by which bioactive chemicals avert apoptosis. The study used HepG2 cells as *in-vitro* models. The inner and outer leaflets of the plasma membrane have asymmetric distributions of phospholipids. The lipid bilayer's external leaflet exposes phosphatidylcholine and sphingomyelin, while phosphatidylserine is exposed by the inner leaflet.

During apoptosis, this asymmetry is broken, exposing phosphatidylserine on the outside surface of the plasma membrane. [30-31]. The amount of LDH present in the culture medium is one indicator used to forecast damage to cell membranes. As predicted, ETHE caused HepG2 cells to release more LDH.

These findings strongly imply that ETHE suppresses the proliferation of Hep G2 cells, most likely due to cell membrane rupture that results in cell death. [32]. In the final part of the study, the apoptogenic effect of ETHE was estimated in cultured human cancer cell lines by Annexin-V/PI staining by flow cytometry analysis. Annexin-V/PI staining was performed to visualize the morphological changes in cells treated with ETHE of *M. rubicaulis* (Lam.). ETHE-treated cells showed nuclear condensation indicating apoptosis. Similarly, the Annexin-V/PI staining assay demonstrated the changes in membrane permeability in ETHE-treated cell lines, indicating apoptosis initiation. These results, demonstrate the ability of ETHE of *M. rubicaulis* (Lam.) to exert apoptosis, particularly in late-stage apoptosis in the HepG2 cell line [33].

In this investigation, it was observed that the percentage of cells dealing with late apoptosis increased dramatically, showing that apoptosis was one of the principal ways of cell death induced by the plant extracts, particularly ETHE on the HepG2 cell line. [34]. As a result, it is postulated that the mechanism by which ETHE of *M. rubicaulis* (Lam.) imparts its anticancer action could be the activation of apoptosis in cancer cells, which could be mediated by the active components found in the ETHE of *M. rubicaulis* (Lam.). [35- 36].

4. Conclusion

In conclusion, *M. rubicaulis* (Lam.) leaf extracts exhibited a cytotoxic effect against HepG2 cancer cells while displaying no toxicity toward L929 normal cells. The outcomes additionally proved that ETHE causes apoptosis in HepG2. Drawing from our findings, we propose that extracts from *M. rubicaulis* (Lam.) leaves could be a promising new therapeutic option for treating liver cancer in humans, provided that they do not exhibit cytotoxicity towards normal cells. The findings of the present investigation demonstrate that the ETHE caused growth inhibition irrespective of the origin of the cell; however, we could observe high cytotoxicity (IC_{50} = 93.69 µg/ml) in cancer cells. Screening of the extracts revealed that the ETHE had shown cytotoxicity could induce apoptosis in cancer cells.

Conflict of interest

The authors declare to have no conflict of interest.

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