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EVALUATION OF ANTICANCER POTENTIAL OF METHANOLIC EXTRACT OF PETALS OF *CHRYSANTHEMUM MORIFOLIUM* AGAINST HUMAN LIVER CANCER CELL LINE (HEPG2)

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Keywords:

MTT Assay, Apoptosis, Cell cycle analysis

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ABSTRACT: *Chrysanthemum morifolium* is commonly known as chrysanthemum. It has been widely used in traditional medicine due to its various therapeutic effects. Past studies have shown that chrysanthemum extracts possess significant anticancer activity against breast, prostate, and colon cancer cells. The active compounds in chrysanthemum extracts such as flavonoids, sesquiterpenes, and triterpenoids have been found to induce apoptosis (programmed cell death) in cancer cells, inhibit cell proliferation thus prevent cancer cell migration and invasion. Furthermore, chrysanthemum extracts have also been reported to enhance the efficacy of conventional chemotherapy and reduce its side effects. Based on the previous studies the present research has been aimed to determine the anticancer activity of petals of *Chrysanthemum morifolium* against Hep G2 liver cancer cells by evaluating the cytotoxic potential by MTT assay ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay), the study of apoptosis mechanism and cell cycle analysis by flow cytometry to understand the cell cycle changes in liver cancer cells when compared with Doxorubicin. The results obtained have demonstrated a prominent anticancer activity of methanolic extract of petals of *Chrysanthemum morifolium* against liver cancer cell lines at IC_{50} 44µg/ml.

INTRODUCTION: The sixth most frequent cancer in the world is liver cancer [1]. In terms of prevalence, it ranks ninth among cancers in women and fifth among cancers in males. Cirrhosis, chronic viral hepatitis, liver fluke infestation, long-term use of oral contraceptives containing high amounts of estrogen and progesterone, and smoking are all known to raise the risk of liver cancer. Strong evidence also exists that being obese or overweight, alcohol consumption increases the risk of liver cancer.

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Consuming foods tainted with aflatoxins (toxins created by a specific fungus) raises the risk of liver cancer, as drinking coffee lowers the risk of developing liver cancer. Some evidence suggests that fish consumption may reduce the risk of liver cancer. Participating in physical activity may reduce the incidence of liver cancer.

The risk of developing hepatocellular carcinoma is higher in patients with cirrhosis (liver scarring as a result of prior injury); 90–95 percent of people with hepatocellular carcinoma have cirrhosis as a contributing factor. Hence any cause of cirrhosis, whether chemical or viral, is likely to raise the risk of developing cancer. Flavonoids and terpenoids are among the various phytochemical constituents possessing a wide variety of activities including anticancer activity for various types of cells. The primary dietary sources of flavonoids include

fruits, vegetables [2], and beverages made from plants, including green tea, wine [3], and products made from cocoa. Flavonoids have been found to have a wide range of anticancer actions, including modulating the activity of enzymes that scavenge reactive oxygen species (ROS), participating in cell cycle arrest, inducing apoptosis and autophagy, and reducing the proliferation and invasiveness of cancer cells. Flavonoids operate as antioxidants under normal circumstances and are strong pro-oxidants in cancer cells, stimulating the apoptotic pathways and down regulating pro-inflammatory signalling pathways. Studies conducted both *in-vivo* and *in-vitro* revealed that flavonoids could have potent anti-inflammatory, immune-modulatory, and anticancer effects [4].

Terpenoids [5] are a vast class of secondary metabolites with isoprenoid units that are present in a wide range of plants. Inhibiting the early initiation and progression of tumorigenesis by inducing cell cycle arrest, tumour cell differentiation, and apoptosis, as well as suppressing angiogenesis, invasion, and metastasis in the late stages of tumour development through the regulation of various intracellular signalling pathways, are just a few of the terpenoids that have been discovered to possess anticancer properties. Antioxidant [6] radicals are unstable atoms that have the potential to harm the body's cellular DNA, which is thought to contribute to the emergence of cancer. When we breathe or exercise, our bodies manufacture free radicals, and we are also exposed to more of them from environmental contaminants like cigarette smoke, air pollution, and UV rays from the sun. They move about the body and can lead to long-lasting inflammation.

Antioxidants function by locating free radicals and counteracting their negative effects. The body's cells are kept healthier and less likely to develop cancer as a result. So, plants exhibiting antioxidant activity are very important to show anticancer activity. The flavonoids, terpenoids, and antioxidant capacity are very much important in a plant extract to reduce cancer cells in our body. *Chrysanthemum morifolium* aqueous methanolic petal extracts were used in this study, and they were put through phytochemical analysis. As a first step in determining IC50 values, the MTT [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide] test was used to assess the cytotoxic potential of *Chrysanthemum morifolium*. To understand the mechanism of cytotoxicity, the observed IC_{50} concentrations were used to study the cell cycle analysis and apoptosis. The findings from each investigation were tabulated and described in the text. *Chrysanthemum morifolium* [7], commonly known as chrysanthemum or garden mum, is a compact, clump-forming, herbaceous perennial that typically grows 2-3' tall. It is native to China. Solitary (infrequently appearing in a loose corymb), creamy yellow flowers (to 2 3/8" across) appear in an often-prolific mid-season bloom extending from September to frost.

Flower colours of various cultivars of this species include shades of white, yellow, orange, lavender, purple, or red. Deeply lobed dark green leaves. The plant has a thick, leathery feel [8]. The numerous silky branches combine to create a thick tuft that is covered in short down. The usual flower heads are radiating, which means they are made up of actinomorphic, tubulated, bisexual center florets and peripheral, female, and zygomorphous florets with ligules. Herbaceous exterior bracts with a little scarcity border are present. Many cup-shaped partial inflorescences are present in complex total inflorescences. The tongue flowers come in a wide range of hues, including green, white, yellow, pink, and purple. There are variations with daisy-like simple blooms and varieties with double flowers that resemble more or less large pompoms. When the day is fewer than 14 hours long, the plant begins to blossom.

To be noted, during the course of the 1.5 millennia of cultivation, tens of thousands of unique cultivars have been produced, with flower heads that varied greatly in terms of their size, color, and shape. The leaves are primarily where one can determine whether something is a chrysanthemum. Overall, our goal is to provide a valuable resource for healthcare professionals and researchers working to improve the prevention, diagnosis, and treatment of liver cancer.

MATERIALS AND METHODS:

Collection of the flowers: *Chrysanthemum morifolium* was purchased from the local market of the Anantapuramu district.

Chemicals: All the chemicals and reagents mentioned in the study were purchased from Sigma-Aldrich (Bengaluru, India).

Preparation of Methanolic Extract: The *Chrysanthemum morifolium* flower petals we separated from the flower stack and cleaned the petals with water followed by methanol. The petals were placed in the 5L conical flask for 48 hours without disturbing it. Then methanol was separated from the petals and placed in the rotary vacuum evaporator [9]. After six hours, solvents were recovered using a rotating vacuum evaporator under reduced pressure. The acquired extract was applied to future investigations.

Phytochemical Screening: Using the following accepted procedures, the methanolic plant extract was examined for the presence of phytochemical constituents [10-13].

Test for Terpenoids: 5 ml of the aqueous plant extract was combined with 2.0 ml of chloroform, which was then added, evaporated on the water bath, and heated with 3 ml of concentrated H_2SO_4. When terpenoids took shape, a grey tint emerged.

Tests for Flavonoids:

Shonda Test: After being combined with aqueous crude plant extract for a short period of time, pieces of magnesium ribbon and concentrated HCl demonstrated that flavonoid was present due to the pink colour.

An Alkaline Reagent Test: A intense yellow hue was created when 2 ml of a mixture of 2.0% NaOH and aqueous plant crude extract was combined together; this colour was neutralized by the addition of 2 drops of diluted acid. The presence of flavonoids was demonstrated by this result.

Test for Steroids: The 5 ml of aqueous plant crude extract was combined with 2 ml of chloroform and concentrated H_2SO_4. The presence of steroids was revealed by the red color that developed in the bottom chloroform layer.

Test for Anthraquinones: 10 ml of benzene was added in 6 g of the plant powder sample in a conical flask and soaked for 10 minutes and then filtered. Further 10 ml of 10% ammonia solution was added to the filtrate and shaken vigorously for 30 seconds and pink, violet, or red colour indicated the presence of anthraquinones in the ammonia phase.

Tests for Glycosides: Liebermann test: Using the full aqueous plant crude extract, we added 2.0 ml of acetic acid and 2 ml of chloroform. After cooling the mixture, concentrated H2SO4 was added. Aglycone, the steroidal component of glycosides, was represented by the color green.

Keller-Kiliani Test: The 10 ml of aqueous plant extract and the 1 ml of concentrated H_2SO_4 were combined with a 4.0 ml solution of glacial acetic acid and a drop of a 2.0% $FeCl_3$ combination. Between the layers, a brown ring formed, revealing the presence of glycosides. Salkowski test: We added 2 ml of concentrated H_2SO_4 to the crude aqueous plant extract as a whole. A reddish-brown hue developed, indicating the presence of the glycoside.

Tests for Alkaloids: The powdered plant material was boiled with 5 ml of dilute sulphuric acid and filtered. Filtrate was used for the following tests.

Dragendroff's Reagent: To the filtrate, a few drops of Dragendroff's reagent were added and shaken well.

Wagner's Reagent: To the filtrate, a few drops of Wagner's reagent were added and shaken well.

Hager's Reagent: To the filtrate, a few drops of Hager's reagent were added and shaken well.

Mayer's Reagent: To the filtrate, a few drops of Mayer's reagent were added and shaken well.

Test for Tannins: The 0.5 g of aqueous extract was combined with 10 ml of bromine water. The presence of tannins was revealed by the decolorization of bromine water.

Test for Saponins: 5.0 ml of distilled water was mixed with aqueous crude plant extract in a test tube and it was mixed vigorously. The frothing was mixed with a few drops of olive oil and mixed vigorously and the foam appearance showed the presence of saponins.

MTT Assay: The HepG2 (Human hepatocellular adenocarcinoma cell line) is purchased from

NCCS, Pune, India. The cells were maintained in DMEM low glucose media supplemented with 10 % FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2, 18-20% O_2 at 37°C temperature in the CO_2 incubator and sub-cultured every 2 days. Passage number-46 was used for the present study. Seeded 200 µl cell suspension in a 96-well plate at the required cell density (20,000 cells per well), without the test agent. Allowed the cells to grow for about 24 hours [14].

The flower extract is taken into 5 concentrations they are (12.5, 25, 50, 50, 100, 200µg) and doxorubicin (3.5µM) is taken for performing the assay. Incubated the plate for 24 hrs at 37°C in a 5% CO_2 atmosphere.

After the incubation period, taken out the plates from the incubator, removed the spent media, and added MTT reagent to a final concentration of 0.5 mg/mL of total volume. Wraped the plate with aluminium foil to avoid exposure to light. Returned the plates to the incubator and incubated them for 3 hours.

(Note: Incubation time varies for different cell lines. Within one experiment, incubation time should be kept constant while making comparisons.) Removed the MTT reagent and then added 100µl of solubilization solution (DMSO). Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals, especially in dense cultures. Read the absorbance on a spectrophotometer or an ELISA reader at 570 nm wavelength. % Cell viability is calculated using below formula:

% Cell viability = Mean abs of treated cells / Mean abs of Untreated cells x 100

The IC_{50} value was determined by using a linear regression equation *i.e.*, Y = mx+c., Y = 50, M and C values were derived from the viability graph [15].

Annexin-V-Fitc Staining Protocol: Cultured cells in a 6-well plate at a density of 0.5 x 10^6 cells/2 ml and incubated in a CO2 incubator overnight at 37°C for 24 hours. Aspirated the spent medium and treated the cells with the required concentration of experimental compounds and controls (Plant extract 44 µg/ml and doxorubicin 3.5 µM) taken in 2 ml of culture medium and incubated the cells for 24 hours. At the end of the treatment, removed the medium from all the wells and given a PBS wash. Removed the PBS and added 200µl of trypsin-EDTA solution and incubated at 37°C for 3-4 minutes. Added 2 ml culture medium and harvested the cells directly into 12 x 75 mm polystyrene tubes. Centrifuged the tubes for five minutes at 300 x g at 25°C. Carefully decanted the supernatant. Washed the cells twice with PBS.

Decanted the PBS completely. Added 5µl of FITC Annexin V. Gently vortexed the cells and incubated for 15 min at RT (25°C) in the dark. Added 5µl of PI and 400µl of 1X Annexin Binding Buffer to each tube and vortexed gently. Analysed by flow cytometry immediately after adding [16].

Cell Cycle Analysis by Flow Cytometry: Cultured cells in a 6-well plate at a density of 2 x 10^5 cells/2 ml and incubated in a CO_2 incubator overnight at 37°C for 24 hours. Aspirated the spent medium and treated the cells with the required concentration of experimental compounds and controls (Plant extract 44µg/ml and doxorubicin 3.5µM) taken in 2 ml of culture medium and incubated the cells for 24 hours.

At the end of the treatment, removed the medium from all the wells and given a PBS wash. Removed the PBS and added 200µl of trypsin-EDTA solution and incubated at 37°C for 3-4 minutes. Added 2 ml culture medium and harvested the cells directly into 12 x 75 mm polystyrene tubes. Centrifuged the tubes for five minutes at 300 x g at 25°C [17]. Carefully decanted the supernatant. Washed with PBS. Decanted the PBS completely.

Fixed in 1ml cold 70% ethanol. Added drop wise to cell pellet while vortexing. This should ensure the fixation of all cells and minimize clumping. Incubated for 30 minutes in -20°C freezer. Pellet cells at a higher speed compared to live cells for 5 minutes, aspirated the supernatant being careful not to lose the pellet. Note that ethanol-fixed cells require higher centrifugal speeds to pellet compared to unfixed cells since they become more buoyant upon fixation. Washed twice with PBS. To ensure that only DNA is stained (PI stains all nucleic acids), treated the cell pellet with 400µL Propidium Iodide/RNase staining buffer.

Mixed well. Incubated cells for 15 to 20 minutes at room temperature in the dark analysed samples by flow cytometry in PI/RNase solution (no need to wash cells) [18].

RESULTS AND DISCUSSION: The chemical tests revealed that plant extract contains various secondary metabolites with various concentrations. Flavonoids and terpenoids were confirmed by various chemical tests. The complete summary of the results of chemical tests for the Methanolic extract of Chrysanthemum morifolium is mentioned in **Table 1**. In the MTT assay seven culture conditions are untreated, Doxorubicin 3.5µM, Extract-12.5µg, Extract-25µg, Extract-50µg, Extract-100µg, Extract-200µg are taken. % cell viability for the seven culture conditions is 100, 43.56, 84.80, 72.92, 45.15, 23.72, 4.00, and IC_{50} was founded to be 44µg/ml. As the concentration of extract increases, there is a decrease in cell viability. The results of the MTT assay are mentioned in **Table 2**.

Extract showed the Apoptosis rate at different conditions of Untreated, Std control, and Extract with 0.74%, 64.46%, and 50.69% respectively. The extract showed significant early and late apoptosis similar to the std drug, Doxorubicin used for the study and may have therapeutic potential against human liver cancer. The results obtained are mentioned in **Table 3.**

% cells inhibited in the different stages of the HepG2 cell cycle. In the Sub G0/G1 phase (Apoptotic phase), 1.42%, 5.8%, and 8.8% of cells get arrested in Untreated, Standard, and Extract with IC_{50} concentration respectively. In G0/G1 phase (Growth Phase), 56.13%, 45.47% and 45.66% of cells get arrested in Untreated, Standard, and Extract with IC_{50} concentration respectively. In the S phase (synthetic phase), 6.79%, 6.67%, and 5.52% of cells get arrested in Untreated, Standard, and Extract with IC_{50} concentration respectively. On the other hand, in the G2/M phase, 35.66%, 42.06%, and 40.02% of cells get arrested in Untreated, Standard, and extract. The results of the cell cycle analysis are mentioned in **Table 4.**

The detailed methods are described above and the results are also given in **Table 1**.

TABLE 1: PHYTOCHEMICAL ANALYSIS FOR THE METHANOLIC EXTRACT OF *CHRYSANTHEMUM MORIFOLIUM*

Phytochemical constituents	Methanolic extract
Terpenoids	+
Flavonoids	+
Steroids	-
Anthraquinones	-
Glycosides	-
Alkaloids	+
Tannins	+
Saponins	+

+ indicates the presence of constituents and - indicates the absence of constituents.

Anti-Cancer Activity:

MTT Assay:

TABLE 2: ABSORBANCES VALUE OF EXTRACT AGAINST HEPG2 CELLS AFTER THE TREATMENT PERIOD OF 24 HRS

Concentration Unit: µg/ml	Incubation: 24hrs		Cell line: HepG2						
Parameter	**Blank**	**Untreated**	**Std control**	**12.5**	**25**	**50**	**100**	**200**	**IC_{50}**
Abs reading 1	0.046	2.069	0.946	1.781	1.561	0.967	0.532	0.106	44µg/ml
Abs reading 2	0.038	2.088	0.912	1.757	1.493	0.956	0.518	0.141	
Mean abs	0.042	2.078	0.929	1.769	1.527	0.961	0.525	0.123	
Mean abs (Sample- Blank)		2.0365	0.887	1.727	1.485	0.919	0.483	0.08	
Standard deviation		0.0134	0.024	0.016	0.048	0.007	0.009	0.024	
Standard error		0.0095	0.017	0.012	0.034	0.005	0.007	0.017	
Cell Viability %		100	43.56	84.80	72.93	45.15	23.72	4.00	

TABLE 3: THE TABLE SHOWED THE % OF CELLS THAT UNDERWENT APOPTOSIS, NECROSIS IN UNTREATED, STD CONTROL, AND THE TEST COMPOUND, EXTRACT TREATED HEPG2 CELLS IN COMPARISON TO VIABLE CELLS

Cell condition	Necrosis	Late apoptosis	Healthy cells	Early apoptosis
Label	UL	UR	LL	LR
Untreated	0.03	0.40	99.23	0.34

Std control	5.93	56.87	29.61	7.59
Extract	18.57	39.54	30.74	11.15

TABLE 4: TABLE SHOWED THE % CELLS ARRESTED IN THE DIFFERENT STAGES HEPG2 CELL CYCLE

% Cells arrested in different cell cycle stages vs HepG2				
Sl. no.	**Cell Cycle stage**	**Untreated**	**Std control**	**Extract**
1	Sub G0/G1	1.42	5.8	8.8
2	G0/G1	56.13	45.47	45.66
3	S	6.79	6.67	5.52
4	G2/M	35.66	42.06	40.02

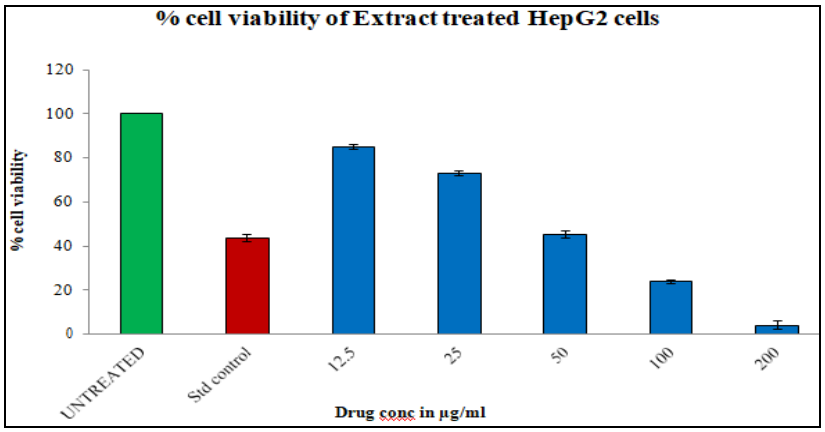


FIG. 1: % CELL VIABILITY VALUES OF EXTRACT TREATED HEPG2 CELLS WITH DIFFERENT CONCENTRATIONS ALONG WITH CONTROLS AFTER THE INCUBATION PERIOD OF 24 HRS

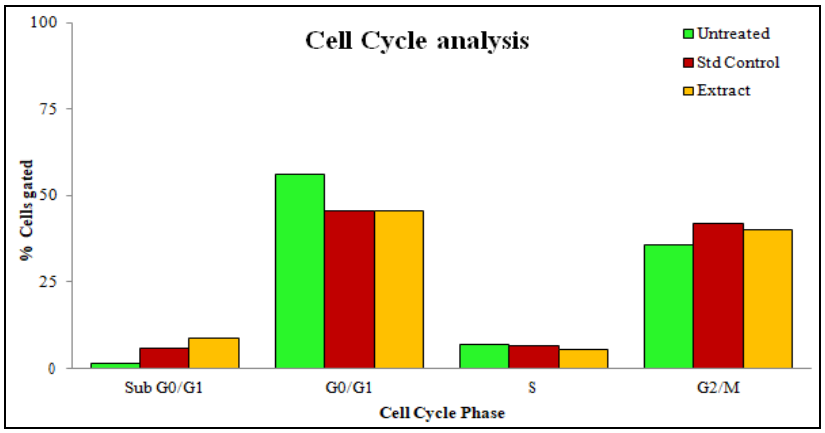
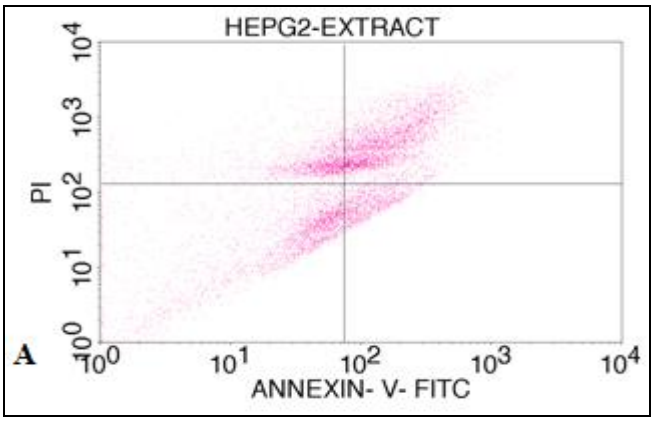


FIG. 2: OVERLAID BAR GRAPH SHOWED THE % CELLS GET ARRESTED IN THE DIFFERENT PHASES OF THE HEPG2 CELL CYCLE

Annexin-V-Fitc Staining Protocol



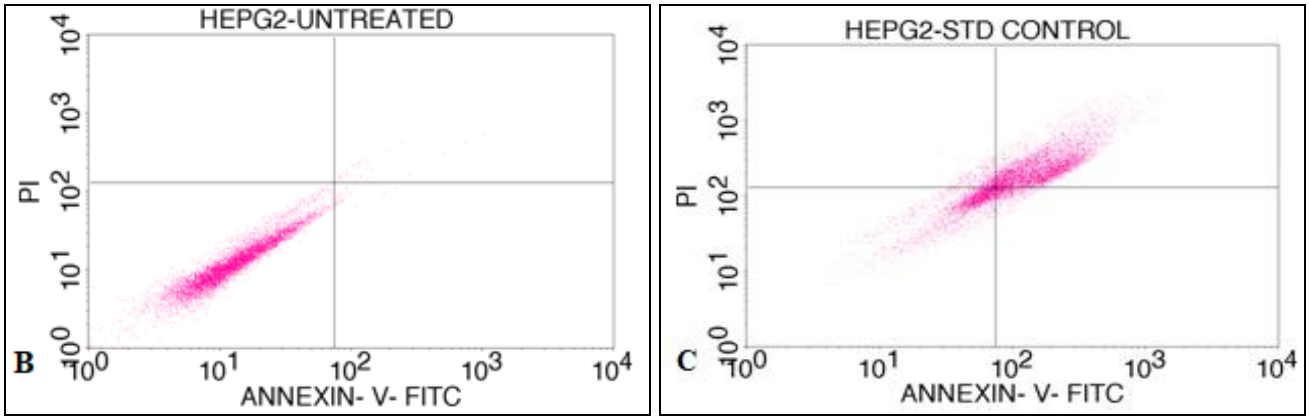


FIG. 3: QUADRANGULAR PLOTS REPRESENTING THE ANNEXIN V/PI EXPRESSION IN HEPG2 CELLS UPON CULTURING IN THE PRESENCE AND ABSENCE OF TEST COMPOUND, EXTRACT WITH IC50 CONCENTRATION ALONG WITH CONTROLS. ANALYSIS WAS DONE BY USING BD FACS CALIBUR, CELL QUEST PRO SOFTWARE (VERSION: 6.0). HERE, ANNEXIN V- FITC - PRIMARY MARKER, PI- PROPIDIUM IODIDE (SECONDARY FLUORESCENCE MARKER) (A-CELL CONTROL, B –STD CONTROL, AND C-EXTRACT WITH 44UG/ML).

UL – Upper left: % of Necrotic Cells	UR - Upper right: % Late Apoptotic Cells
LL- Lower left: % Viable Cells	LR- Lower right: % of Early apoptotic cells

Cell Cycle Analysis by Flow Cytometry:

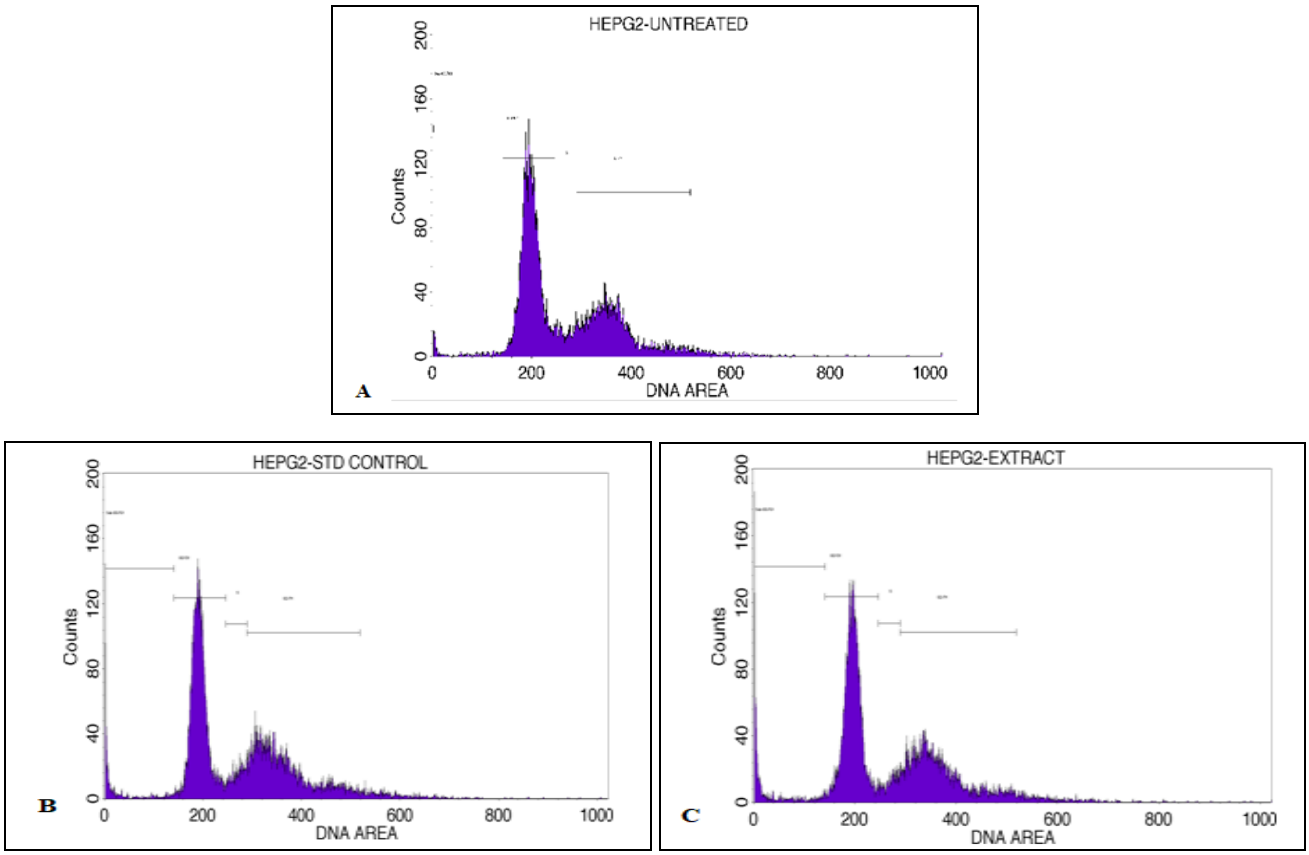


FIG. 4: FLOW CYTOMETRIC HISTOGRAMS SHOWED THE PHASES OF CELL CYCLE DISTRIBUTION IN THE HEPG2 CELL LINE TREATED WITH THE TEST COMPOUND, EXTRACT WITH IC_{50} VALUE, AND STANDARD DRUG, DOXORUBICIN WITH 3.5UM/ML CONCENTRATION COMPARED TO THE CONTROL. (A-Cell Control, B –Std Control, and C-Extract with 44ug/ml).

CONCLUSION: Presence of flavonoids and terpenoids is confirmed by the chemical test. Flavonoids and terpenoids have the capacity to reduce cancer activity. The results of the

cytotoxicity study performed by MTT assay suggest that the test compound, Extract was cytotoxic in nature on Human liver cancer (HepG2) cells with the IC_{50} value of 44µg/ml. The observations suggested us that the test compound, Extract induced effective apoptosis in human liver cancer cells used for the study. The cells treated with Std Control and the test compound, extract with IC_{50} concentration showed a high % of cells at the G2/M phase similar to the Std control, Doxorubicin used for the study. Hence the cell cycle got arrested at G2/M and Sub G0/G1 phases. Given compound exhibited prominent Cell Cycle phase arrest similar to the std Control, Doxorubicin on HepG2 cells and confirmed the anti-liver cancer effect.

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