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PHARMACOLOGICAL INVESTIGATION IN ANTICANCER AND ANTIOXIDANT PROPERTY OF *CHRYSANTHEMUM MORIFOLIUM* RAMAT.

Maliha Munawar[1], Sana Javaid Awan[1], Alina Farooq[1], Mubeen Shahzadi[1,3], Bushra Adeel[1,3] and Haleema Usman Khan[1]

[1]Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Pakistan.
[2]The Medical Laboratories private Ltd, Lahore. Pakistan.
[3]Rashid Latif Medical College, Lahore. Pakistan

***Corresponding Author:** Dr. Sana Javaid Awan (PhD)
*1km Off Bhupptian Chowk, Raiwind Road Lahore. The University of Lahore, Lahore, Pakistan,
Email: sana.javaidawan@yahoo.com, sanajavaiduol@gmail.com, sana.javaid@imbb.uol.edu.pk,
Tel: 092-3224866719.

Abstract
Chrysanthemum morifolium Ramat., commonly referred to as the decorative chrysanthemum or simply mum, has served ornamental and therapeutic objectives historically. Traditionally employed to manage inflammation, fever, and hypertension, modern exploration has paid particular focus to its conceivable function as an antineoplastic and antioxidant ingredient. Its bioactive mixtures, incorporating flavonoids and polyphenols, have proven potential in discouraging malignant cell proliferation, stimulating apoptosis, and bettering antioxidant safeguards. Various tactics were utilized in the research to investigate CM's pharmacological impacts. Plant extracts were ready using ethanol, and their cytotoxicity was evaluated using cell viability assays (MTT, Crystal Violet, Trypan Blue). IC50 values were calculated for HepG2 and HeLa cancerous cell lines. Moreover, antioxidant properties were assessed using catalase (CAT), ascorbate peroxidase (APOX), and glutathione reductase (GSH) assays. Enzyme-linked immunosorbent assays (ELISA) measured fluctuations in p53 and VEGF levels. CM revealed dose-dependently cytotoxic impacts against HepG2 and HeLa cells, with notable reductions in cell viability and heightened cell death seen at elevated concentrations. IC50 values indicated powerful antineoplastic action while sparing noncancerous 3T3 cells. Antioxidant assays uncovered enhanced CAT, APOX, and GSH activities in cancerous cells treated with CM, proposing a role in oxidative stress supervision. Chrysanthemum morifolium Ramat. puts forth encouraging anticancer properties through selective cytotoxicity and apoptotic induction in cancerous cells, backed by heightened antioxidant defenses. Its safety profile in normal cells emphasizes its potential as a therapeutic agent with diminished side effects. However, further exploration is necessary to elucidate the underlying molecular mechanisms and validate their efficacy in animal models and clinical trials. These discoveries underscore CM as a candidate for developing targeted cancer therapies and enhancing overall human health.

Keywords: *Chrysanthemum morifolium* Ramat., angiogenesis, medicinal plants, anticancer and anti-oxidants.

Introduction

Chrysanthemum morifolium Ramat. is widely used as an ornamental and medicinal plant, commonly called Florist's chrysanthemum or Mum (A. Khan, Choudhury, Khanal, & Maukeeb, 2021; A. U. Khan, Choudhury, Khan, Khanal, & Maukeeb, 2020). Chrysanthemum has been widely used in traditional medicinal systems as a remedy for various ailments like inflammation, fever and hypertension. The newly investigated antineoplastic and antioxidant effect of *Chrysanthemum morifolium* Ramat. has attracted significant interests in pharmacological research (Hadizadeh, Samiei, & Shakeri, 2022; Maddala, 2021).

Natural compounds with antineoplastic properties shows important role in the discovery of new cancer treatments (Dehelean et al., 2021; Garcia-Oliveira et al., 2021). Cancer is a phenomenon of random abnormal cell growth and division then why cancer are still killing a majority of people on earth. Chemotherapy, radiation and the like can have terrible side effects and are ineffective because of drug resistance (Compton & Compton, 2020; Current, 2020). Understanding plant-derived compounds, therefore, provides an optimistic addition or option for enhancing cancer therapeutics (Amin et al., 2021; Singh, Singh, Kharwar, White, & Gond, 2021). *Chrysanthemum morifolium* Ramat., a well-known Chinese medicinal herb, contains various bioactive compounds (flavonoids and polyphenols) that were reported to exert potential anti-cancer activities such as proliferation inhibition, apoptosis induction and suppression of metastasis in several cancer cell lines according to previous studies (Liu et al., 2024; Mohamad & Zahari, 2024; Ojha, Vishwakarma, Tripathi, & Mishra, 2024).

Chrysanthemum morifolium Ramat., with the potential antineoplastic effect, is also known for antioxidant activity as well. Introduction Oxidative stress (OS), where there is an imbalance between reactive oxygen species (ROS) and the capacity of the biological system to detoxify these reactive intermediates, is critical in the pathogenesis of chronic diseases such as cancer (Ng, Loh, Tan, Tan, & Wee, 2020; Yuan et al., 2020). ROS; free radicals) and prevent the damage done to the cells (antioxidants), hence helping to prevent disease *Chrysanthemum morifolium* Ramat., with the highest content of phenolic compounds, might be a potential natural antioxidant source as it may correlate to cancer chemoprevention and promotion of human health (Doan et al., 2024; Hodaei, Rahimmalek, & Arzani, 2021).

Although these are encouraging qualities, but there are still different lacunae in the available research on antineoplastic activities and antioxidants of *Chrysanthemum morifolium*. Present in-depth corresponding mechanism of the biological effects of *Chrysanthemum morifolium* Ramat. is not clear. This article provides insight of bioactive components and their action as antineoplastic, antioxidant in *Chrysanthemum morifolium* Ramat. However, in order to explore this possibility fully, requires in depth in vivo studies, mechanistic studies and clinic trials. This may help to be fully aware of new possible therapeutic options of *Chrysanthemum morifolium* Ramat.

Methodology

Preparation of Plant Extracts

Collection of leaves were done for the understudy medicinal plant *Chrysanthemum morifolium* Ramat. in accordance to WHO, 2004. Extracts get chipped to leave powdery forms that dry later on. For ethanolic extract the plant materials (200g) were taken in stoppered container containing 600 ml of methanol and kept on a shaker at normal temperature for duration of 7 days. The extract was then taken in lyophilized forms and stocked solutions were prepared at 20mg/ml in dimethyl sulfoxide (DMSO) from stock procured. This was applied to each combination in triplicate against cell lines.

Culturing of Cell Lines

All cryovials were de-frosted by incubation at 37 degrees from liquid nitrogen cylinders. HepG2, HeLa, and 3T3 cell lines were cultured in cell culturing flask and added DMEM and 10% fetal bovine serum (FBS) supplemented by 100mg/mL penicillin of G (Sigma) and 100 U/mL of streptomycin (Sigma). All the cells were cultured in a humidified incubator with 5% CO2 at 37˚C, as already described, and experiments performed thrice in replicates. HepG2, HeLa, and 3T3 cells were sub-

cultured at up to 70-80% confluency in culture plates. Upon splitting, cells that had adhered by walls of the culture flask were thoroughly cleaned with 1X (PBS) and with 0.05% trypsin-EDTA it was incubated till the cells were removed from the surface of culturing flask. Which was confirmed after viewing the flask on inverted microscope. Media was collected and a little amount of FBS were added to the flask and vigorously mixed by pipetting. The cells centrifuged for 5 mins at 2000 rpm, the best crystal solution layer was removed and transferred to a fresh Eppendorf. They were centrifuged, and removed supernatant and pellet was re-suspended.

Treatment of Cell Lines with Plant Extract

Cells were seeded on 96-well culture plate (Specifically for cellular viability) and four groups for each kind of cells, HeLa, HepG2 and 3T3 cell lines. One group was un-treated (UT) Control was Cisplatin: HepG2 (HG-UT), HeLa (HL-UT), 3T3 (3T3- UT). Each cell line was given treatment with ethanolic extract of chrysanthemum in various concentrations (50ug/ml, 100ug/ml, 200ug/ml, 300ug/ml and 500ug/m) plant extract in medium DMEM. Cells then were treated for 24 h for in cultures. Cells in 96 well plates were treated with post-treated IC50 after 24 hours. Standard drug that is anti-cancer was Cisplatin. Cells that were post-treated from 96 well culture plates were employed for both crystal violet and trypan blue assay, respectively using the same technique. The secretome from post-treated groups was used for ELISA of p53 and VEGF.

Cell Viability Assays

To estimate cellular viability for post-treated (HepG2, HeLa, and 3T3) cell lines 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT (Invitrogen Inc., USA), crystal violet and trypan blue assays were carried out to evaluate the effects of variable amounts of chrysanthemum ethanolic extract on cells harvested in 96 wells plates.

IC50 Calculation via MTT

Following a 24-hour treatment with varying doses of plant extracts, cells were rinsed with phosphate buffer saline (PBS) from Invitrogen Inc. (USA) and subsequently cultured for 3–4 hours with 100µl DMEM and 25µl MTT solution. After four hours of incubation, 10% sodium dodecyl sulphate (SDS) from Invitrogen Inc. in the USA was used to solubilize the formazan crystals, and the absorbance at 570 nm was measured. The formula used to compute this outcome, known as the % viability, is abs, which stands for absorbance. IC50 values were computed three times for three different tests. Rather, all extracts' IC50 values were applied to later studies.

Dead Cells Detection (Trypan Blue Assay)

The proportion of cells that are dead counted by Trypan blue dye exclusion analysis for live versus dead cells staining. PBS was removed, and different cell lines from corresponding experimental group HeLa, HepG2 or 3T3 were cleaned with PBS by washing for 3 times followed by incubation in Trypan blue (Invitrogen Inc., USA) for 5 min. And the cells were cleaned by PBS for 3 times and observed in a microscope.

Lactate Dehydrogenase Assay (LDH)

All experimental groups had their LDH activity of the cancer cells HepG2, HeLa, and 3T3 in the medium assessed in accordance with the manufacturer's instructions (AMP Diagnostics; Austria). Each experimental group's secretome (5µl) was used for the LDH assay. In a 96-well plate, 5 µl of each group's cell culture medium and 100 µl of working reagent were combined and incubated for 5 minutes. A spectrophotometer set to record absorbance at 340 nm was used.

Live Cells Detection (Crystal Violet Assay)

Crystal violet staining was used to evaluate the viability of experimental groups in a HepG2, HeLa and 3T3 cell lines. The experiment was carried out in 96-well plate. The secretome was removed from the plate wells, and cells were cleaned with PBS by washing. Then equilibrate and 0.1 percent

of crystal violet was added in bottom of wells and incubated on room temperature for 20 minute after washing, last place were washed again, then mix the same this time with at the same volume with %2 ethanol so that it covers all wellernes surface; Room temperature for 15 minutes was used to incubate it. Carefully remove the dye and wash the well with great care to ensure that the cells do not lift off from the well. 1%SDS prepared in 100µl added in each well to solubilize the stain then incubated for 5 to10 minutes. Finally, at 595nm absorbance was checked.

Enzyme-linked Immunosorbent Assay (ELISA)

The secretome and cellular lysate of the post-treated cells were subjected to a solid-phase sandwich ELISA. The following publications from the literature were used to perform anti-VEGF (Santa Cruz Biotechnology, USA) and anti-p53 ELISA from the Secretome of HeLa, HepG2, and 3T3 cell line. Their cellular lysate was employed in a 96-well plate (Corning, USA). To summarise, the plate was coated with antibody for one night at 4°C, followed by washing (1X TBS-T) for five minutes. Next, blocking solution (200µl of BSA/well) was applied to the plate wells, and the plate was incubated for thirty minutes. The blocking solution was then discarded following incubation. 200 µl of secretome from three different experimental groups were added to each well, and after eighteen hours, the wells were washed. Following washing, 100 µl of HRP-conjugated donkey anti-rabbit IgG (SCBiotech, USA) secondary antibody was added to each well. After that, the plates were incubated for the full night at 4°C, which eliminated the secondary antibodies. Following washing, the mixture was incubated for 20 minutes with 100 µl of a solution of the chromogenic substrate 3,3',5,5'-Tetramethylbenzidine (TMB) (Invitrogen Inc., USA) added. Following the addition of 100 microliters of 0.18 M H_2SO_4 stop solution, the absorbance at 450 nanometers was measured.

Evaluation of Antioxidative Enzymes

Glutathione Reductase (GSH) Assay

The Glutathione Reductase (GSH) assay was performed in a 96-well plate format with 200µl final reaction volumes per well, following the GSG concentration methodology. A reaction mixture comprising 40 mM EDTA, 10 mM oxidized glutathione, and 20 mM KH_2PO_4 buffer (pH 7.5) was utilized. In a different experiment, HeLa was used to create two groups of samples: one for pre-treatment and the other for posttreatment. HepG2 and 3T3 cell lines were used to isolate secretome in the reaction mixture. After preparing and treating the nitrate standard (0.5 to 1 ppm) similarly, 20 mM NADPH was added, and the absorbance was measured in a spectrophotometer at 340 nm.

Catalase (CAT) Assay

The catalase activity was evaluated in a 96-well plate, (Shamim and Rehman., 2015), with the secretomes of the HeLa, HepG2, and 3T3 treatment groups, 12.5 mM KH_2PO_4 (pH 7.0), and 31.25 mM H_2O_2 in each well. The optical density of each experimental group, which was incubated in a dark chamber for 45 to 60 seconds, was measured at 240 nm in a well plate relative to a blank.

Superoxide Dismutase (SOD) Assay

In a 96-well plate, superoxide dismutase (SOD) activity was measured using (Shamim and Rehman., 2015). To prepare for this test, secretomes from separate experimental groups (pre- and post-intreat) were mixed with 100 mM KH_2PO_4 buffer (pH7.8), 0.1 mMEDTA, 13 mM methionine, 2.25 mM nitro-blue tetrazolium chloride (NBT), and 60µM riboflavin on Hela, HepG2, and 3T3 cells. The OD at 560 nm was found using the spectrophotometer.

Ascorbate Peroxidase (APOX) Assay

A comparable process using previously published (Shamim and Rehman., 2015) was also carried out, and HepG2, and HeLa, and 3T3 were tested using the APOX assay in 96-well plates. The catalase assay reaction mixture included 2.5 mM ascorbate, 75 mM H_2O_2, and 25 mM KH_2PO_4 buffer (pH 7.0). Furthermore, it contained the secretomes from several experimental groups. three minutes of exposure to light, after which the optical density at 290 nm is measured.

Statistical Analysis

In the experimental groups, all unitary data were presented as mean ± SEM (n = 3). For the statistical analysis of the data, one-way ANOVA was used to compare group means, and Bonferroni's q test was used to evaluate differences between groups. The two-way ANOVA function of the graph pad software was used to perform the statistical analysis of the quantitative data from the various experimental groups. The statistical analysis yielded a significant p-value of less than 0.05. Endnote was used to insert the references.

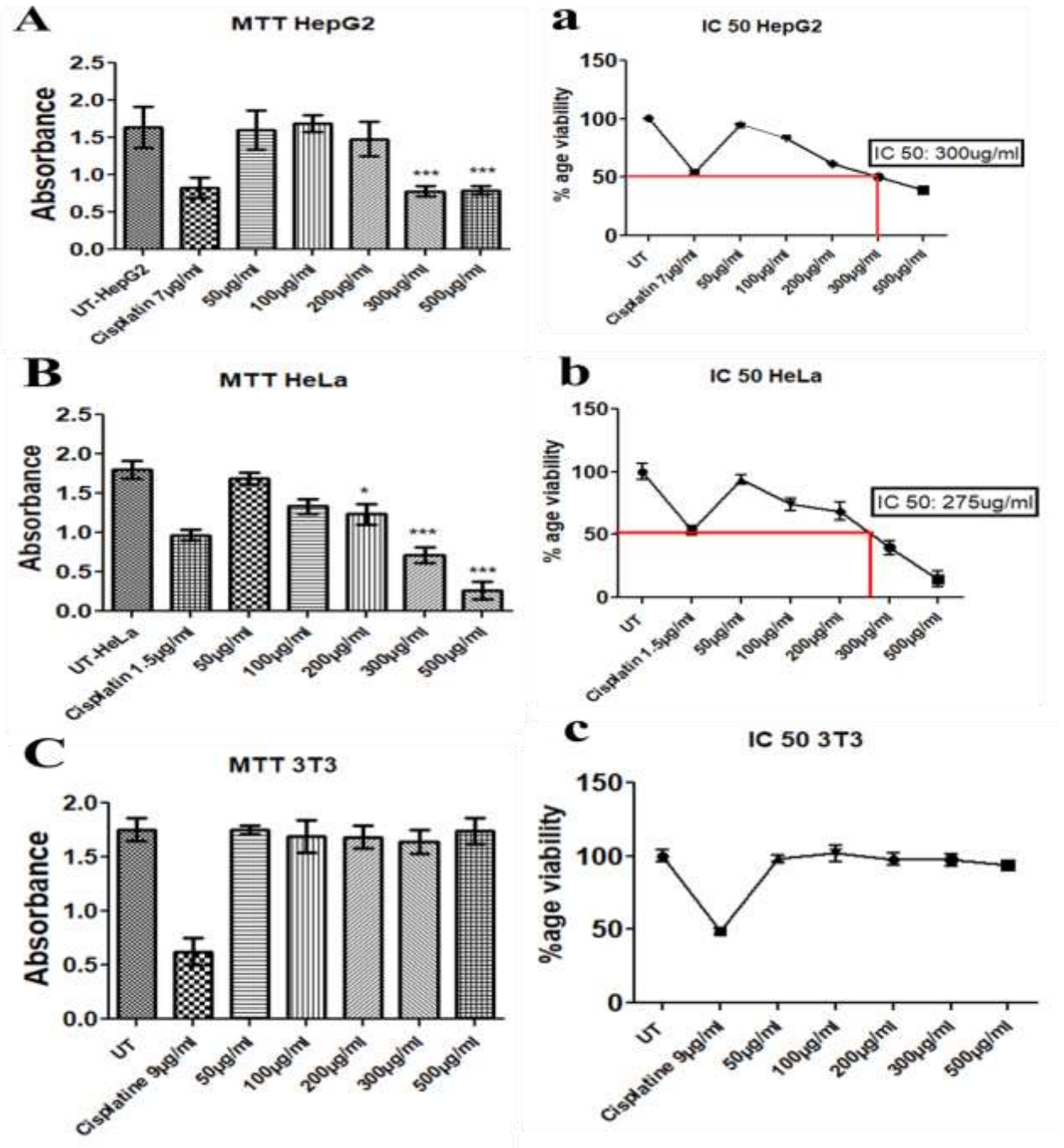
RESULTS

Increased Cytotoxicity of *Chrysanthemum morifolium* Ramat. Extracts

The MTT assay was conducted to evaluate the cytotoxic impacts of *Chrysanthemum morifolium* Ramat. (CM) on both HepG2 and HeLa cells across a variety of concentrations. Testing was carried out to assess the cytotoxic consequences of *Chrysanthemum morifolium* Ramat. (CM) on HepG2 cells across a broad spectrum of dosages as shown in Figure 1(A, a). The absorbance value for untreated HepG2 cells was (1.64±0.275), denoting the standard level of cellular viability. The typical medication, Cisplatin at 7 µg/ml, significantly decreased the absorbance to (0.827±0.142), underlining its powerful toxicity. Treatment with CM at 50 µg/ml displayed minimal decline in absorbance (1.60±0.259), and an growth was spotted at 100 µg/ml (1.69±0.116). However, a concentration-dependent fall in cellular endurance was discerned at higher degrees of CM: 200 µg/ml (1.48±0.229), 300 µg/ml (0.780±0.0666), and 500 µg/ml (0.0666±0.0549). These revelations indicate that CM exhibits significant cytotoxic impacts at more exalted concentrations. The half-maximal inhibitory focus (IC50) was ascertained to be 300 µg/ml, signifying the dosage at which CM chops the viability of HepG2 cells by 50%.

The absorbance readings of untreated HeLa cancer cells were relatively high at (1.80±0.115), signifying their baseline viability within the experiment. However, treatment with the standard anticancer drug cisplatin at just 1.5 µg/ml drastically reduced absorbance to a markedly lower (0.967±0.0677), demonstrating its potent cytotoxic abilities. CM administered at the relatively mild concentration of 50 µg/ml only slightly decreased absorbance to (1.69±0.0700), yet absorbance progressively diminished more conspicuously at higher dosages of 100 µg/ml (1.33±0.0882), 200 µg/ml (1.24±0.130), 300 µg/ml (0.713±0.0984), and the sizeable amount of 500 µg/ml (0.263±0.111). These divergent absorbance results across the range of CM concentrations, as illustrated in Figure 1(B, b), evidently exhibit its dose-dependent decline in viability within the HeLa cells. Intriguingly, the half-maximal inhibitory concentration was precisely calculated to be 275 µg/ml, uncovering that CM exerts meaningful cytotoxic actions against HeLa cells at this substantial dosage.

The MTT assay was also applied to evaluate the toxic impacts of *Chrysanthemum morifolium* Ramat. (CM) on regular 3T3 fibroblasts across an array of concentrations. The absorbance reading for untreated 3T3 cells was relatively consistent at (1.75±0.104), characterizing their baseline survival. Yet cisplatin administered at the rather small amount of 9 µg/ml precipitously dropped absorbance to a remarkably lower (0.627±0.128), underscoring its robustly deadly effects. However, treatment with CM at the relatively small dosages of 50 µg/ml (1.76±0.0405), 100 µg/ml (1.69±0.153), 200 µg/ml (1.69±0.107), 300 µg/ml (1.64±0.110), and 500 µg/ml (1.75±0.121) did not considerably deviate the absorbance readings from the control group Figure 1(C, c). These divergent outcomes infer that CM, across the spectrum of dosages tested, does not exhibit toxic impacts on 3T3 fibroblasts, as cell viability remained comparatively constant and akin to the untreated cells. This signifies a lack of meaningful antiproliferative actions of *Chrysanthemum morifolium* Ramat. on 3T3 fibroblasts, proposing it may be innocuous to regular noncancerous cells at these dosages. These findings expose that *Chrysanthemum morifolium* Ramat. possesses promising anticancer properties.



Figures 1: MTT assay and IC 50 values of *Chrysanthemum morifolium* Ramat. (CM). A) Cell viability of post treated HepG2 cells, a) IC50 calculations of post treated HepG2 cells. B) Cell viability of post treated HeLa cells, b) IC50 calculations of post treated HeLa cells. C) Cell viability of post treated 3T3 cells, a) percentage viabilities of post treated 3T3 cells. Absorbance values and percentage viability for untreated cells and standard drug, Cisplatin were depicted. Values are expressed as mean ± SEM. Where $p<0.05$ is considered as significant.

Decreased Viability of Chrysanthemum Extracts

Crystal violet Assay (Live Cell detection)

The Crystal Violet Assay provided insight into the cytotoxic impacts of *Chrysanthemum morifolium* Ramat. extract on HeLa carcinoma cells across changing concentrations. Initial experiments on HepG2 cells demonstrated a baseline viability of 0.327±0.0176. Cisplatin, the typical pharmacological agent at 7 µg/ml, significantly decreased absorbance to 0.173±0.0145, highlighting its powerful anti-proliferative properties. CM treatment induced dose-dependent reductions in

absorbance: 50 µg/ml (0.247±0.0260), 100 µg/ml (0.160±0.0265), 200 µg/ml (0.120±0.0153), 300 µg/ml (0.0833±0.0120), and the maximal 500 µg/ml concentration (0.0767±0.0233).
Untreated HeLa cells exhibited an absorbance of 0.443±0.0233, serving as the control for cellular vitality. Cisplatin at 1.5 µg/ml potently diminished absorbance to just 0.220±0.0115. CM induced concentration-dependent decreases in absorbance values: 50 µg/ml (0.317±0.0328), 100 µg/ml (0.293±0.0549), 200 µg/ml (0.163±0.0273), 300 µg/ml (0.150±0.0289), with the greatest reduction at 500 µg/ml (0.0567±0.0176). These effects indicate CM significantly depletes HepG2 and HeLa cell viability at higher doses, with maximum cytotoxicity at 500 µg/ml. *Chrysanthemum morifolium* Ramat. thus exhibits notable anticancer properties against HepG2 and HeLa cells, with efficacy amplified at greater concentrations, supporting exploration as a potential therapeutic for liver malignancies. Testing on 3T3 normal fibroblasts exposed no substantial reduction in viability across CM doses from 50 to 500 µg/ml, unlike the powerful cytotoxicity of Cisplatin used as a benchmark.

Trypan Blue Assay (Dead cell deduction)

The Trypan Blue Assay was conducted to assess the cytotoxic impacts of *Chrysanthemum morifolium* Ramat. (CM) on HeLa cancer cells at differing concentrations. The absorbance value for untreated HepG2 cells was (19.1±0.551), representing the baseline level of cell viability. Cisplatin, the standard drug at 9 µg/ml, significantly elevated the absorbance to (45.4±0.516), indicating a notable amount of cytotoxicity. CM treatments demonstrated a dose-reliant increase in cell death: 50 µg/ml (21.9±0.577), 100 µg/ml (23.5±0.574), 200 µg/ml (23.7±0.680), 300 µg/ml (50.2±2.99), and 500 µg/ml (76.4±3.51).
At the same time, the Trypan Blue Assay evaluated the effects of CM on 3T3 normal cells across various concentrations. The reference absorbance value for untreated 3T3 cells was 29.4±0.970, signifying baseline cell viability. Cisplatin, administered at 9 µg/ml as the standard, significantly raised the absorbance to 49.5±0.858, illustrating its cytotoxic properties. In contrast, CM treatment at 50 µg/ml (29.4±0.970), 100 µg/ml (29.2±0.924), 200 µg/ml (28.5±0.659), 300 µg/ml (28.5±0.659), and 500 µg/ml (29.2±0.802) generated no substantial increase compared to the control absorbance values.
These analyses demonstrate that CM induces considerable cytotoxicity in HeLa cancer cells in a dose-dependent manner, with the most potent effects at 500 µg/ml. Meanwhile, CM exhibited no harmful impacts on normal 3T3 cells across all tested concentrations. Therefore, *Chrysanthemum morifolium* Ramat. possesses potent anti-tumor activity selectively against cancerous cells without toxicity towards healthy ones.

LDH ASSAY

The LDH assay was conducted to evaluate the cytotoxic effects of varying concentrations of *Chrysanthemum morifolium* Ramat. extract on HepG2 and HeLa cancer cell lines. Untreated HepG2 cells released LDH at a baseline level of 0.350±0.0709 absorbance units. Cisplatin, used as a cytotoxicity standard at 9 µg/ml, significantly reduced LDH release to 0.220±0.0361, demonstrating its effects. CM treatment increased LDH release concentration-dependently: 50 µg/ml (1.51±0.0410), 100 µg/ml (1.48±0.0384), 200 µg/ml (1.67±0.0338), 300 µg/ml (1.79±0.0406), 500 µg/ml (1.97±0.0273). For untreated HeLa cells, LDH release was 0.517±0.0348 absorbance units. Cisplatin at 9 µg/ml significantly lowered LDH to 0.247±0.0296, reflecting cytotoxicity. CM increased LDH release concentration-dependently as well: 50 µg/ml (1.51±0.0296), 100 µg/ml (1.40±0.0318), 200 µg/ml (1.67±0.0173), 300 µg/ml (1.80±0.0426), 500 µg/ml (1.97±0.0219). These results indicate CM induces cytotoxicity in HepG2 and HeLa cells in a dose-dependent manner, correlating increased LDH release with membrane damage and cell death. The marked LDH elevation at 300 and 500 µg/ml underscores CM's potent cytotoxic effects.
The LDH assay also evaluated CM's cytotoxic effects on 3T3 normal fibroblasts. Untreated 3T3s released LDH at 0.350±0.0709 absorbance units. Cisplatin at 9 µg/ml significantly lowered LDH to 0.223±0.0353, reflecting its cytotoxicity. CM increased LDH release concentration-dependently: 50 µg/ml (1.68±0.0351), 100 µg/ml (1.68±0.0384), 200 µg/ml (1.71±0.0561), 300 µg/ml (1.81±0.0437),

500 µg/ml (1.85±0.0754) in Figure 2(G, H and I). These findings indicate CM induces cytotoxicity in 3T3 cells in a dose-dependent manner correlated to increased LDH release, suggesting membrane damage and cell death as shown in Figure 2(G, H and I). The marked LDH elevation at 300 and 500 µg/ml concentrations highlights CM's significant cytotoxic effects on 3T3 normal fibroblasts.

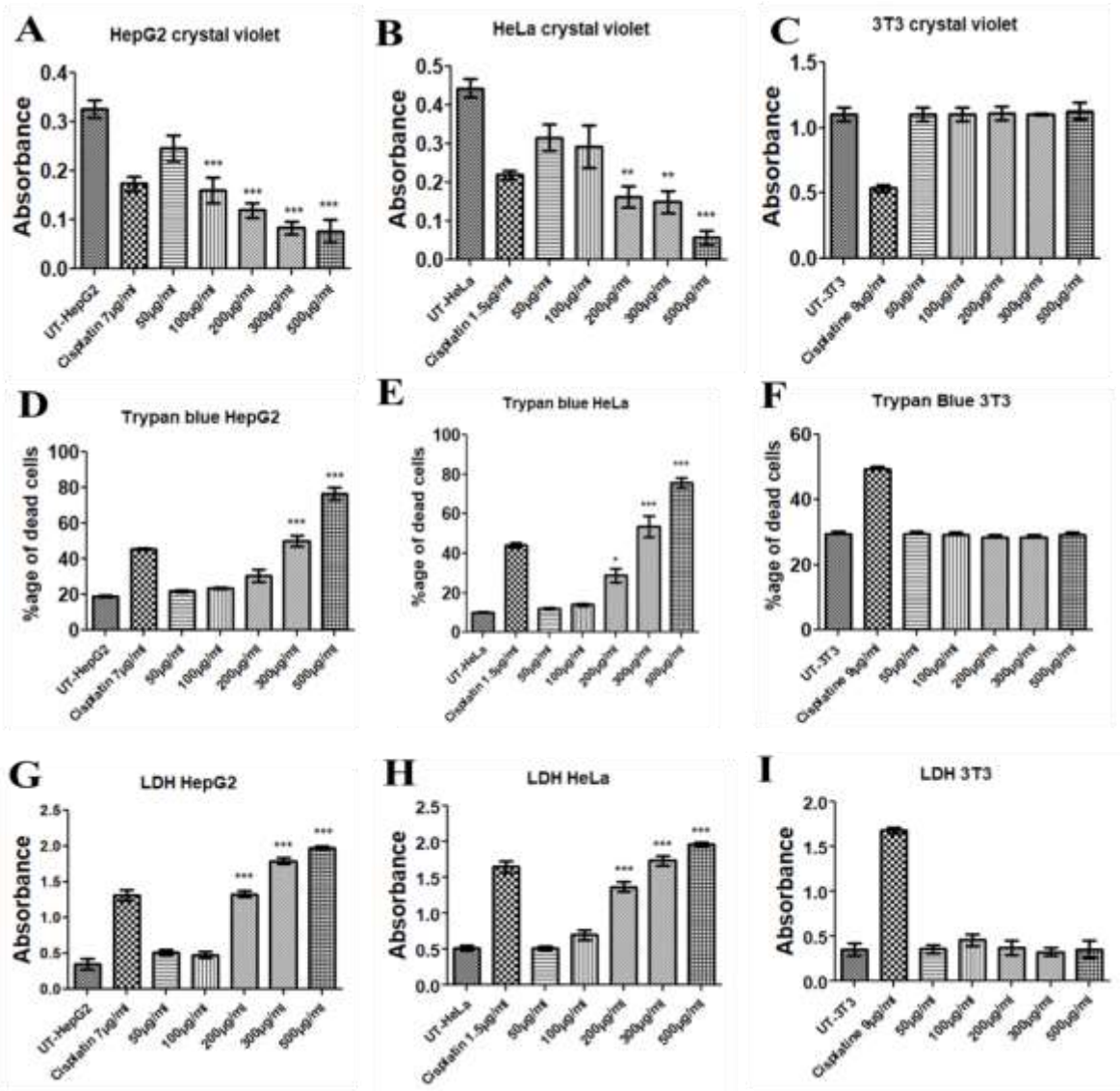


Figure 2: Live cell and dead cell detection of *Chrysanthemum morifolium* Ramat. (CM). A) Live cell detection in HepG2 cells with crystal violet. B) Live cell detection in HepG2 cells with crystal violet. C) Live cell detection in 3T3 cells with crystal violet. D) Dead Cell detection in HepG2 cell line with trypan blue. E) Dead Cell detection in HeLa cell line with trypan blue. F) Dead Cell detection in 3T3 cell line with trypan blue. G) Cell damage detection in HepG2 cell line with LDH. H) Cell damage detection in HeLa cell line with LDH. I) Cell damage detection in 3T3 cell line with LDH. Absorbance values and percentage viability for untreated cells and standard drug, Cisplatin were depicted. Values are expressed as mean ± SEM. Where $p<0.05$ is considered as significant.

Reduced Angiogenic Potential of Chrysanthemum Extracts

The ELISA assay demonstrated varying levels of Vascular Endothelial Growth Factor (VEGF) secreted by HepG2 and HeLa cancer cells following treatment with assorted concentrations of *Chrysanthemum morifolium* Ramat. (CM). Untreated HepG2 cells displayed an absorbance value of (1.82±0.0285), signifying substantial VEGF expression. Cisplatin, the standard drug at 9 µg/ml, considerably diminished VEGF levels, registering an absorbance of (0.630±0.0896). Exposure to CM

yielded a dose-dependent decline in VEGF secretion: 50 µg/ml (1.54±0.0841), 100 µg/ml (1.35±0.0857), 200 µg/ml (1.26±0.0273), 300 µg/ml (1.13±0.140), and 500 µg/ml (1.21±0.139). Untreated HeLa cells manifested an absorbance value of (1.83±0.0351), indicating abundant VEGF expression. Cisplatin, administered as the standard drug at 9 µg/ml, significantly reduced VEGF levels, with an absorbance of (0.630±0.0896). Treatment with CM instigated a concentration-dependent decrease in VEGF expression: 50 µg/ml (1.58±0.0555), 100 µg/ml (1.39±0.0549), 200 µg/ml (1.27±0.0285), 300 µg/ml (1.03±0.0404), and 500 µg/ml (1.10±0.0639). These data suggest CM effectively mitigates VEGF levels in HepG2 cells in a dose-dependent manner, with notable reductions observed at higher concentrations. The study demonstrates that *Chrysanthemum morifolium* Ramat. may inhibit angiogenesis in HepG2 and HeLa cells by diminishing VEGF expression, highlighting its potential as an anti-cancer agent. The absorbance value for untreated 3T3 cells was (1.86±0.0617), indicating baseline VEGF expression. Treatment with Cisplatin, the standard drug at 9 µg/ml, significantly reduced VEGF levels, with an absorbance of (0.630±0.0896). In contrast, treatment with CM revealed minimal variations in VEGF levels across various concentrations: 50 µg/ml (1.58±0.0555), 100 µg/ml (1.56±0.0603), 200 µg/ml (1.58±0.0555), 300 µg/ml (1.55±0.0751), and 500 µg/ml (1.57±0.0493). These results in figure 3(A, B and C) suggest that CM does not appreciably alter VEGF expression in 3T3 normal cells, maintaining levels akin to the untreated control. This indicates that *Chrysanthemum morifolium* Ramat. is non-toxic to normal cells with respect to VEGF expression, highlighting its potential selectivity and safety as a therapeutic agent.

Enhanced Apoptotic Effect of Chrysanthemum Extracts

The ELISA assay was conducted to evaluate p53 protein levels in HeLa cells treated with varying concentrations of *Chrysanthemum morifolium* Ramat. Baseline p53 expression in untreated HepG2 cells registered at an absorbance of 0.680±0.151. Cisplatin, administered at the standard 9 µg/ml dose, significantly boosted p53 to 1.58± 0.0584 as anticipated from its induction of DNA damage and activation of p53 pathways. CM treatment elicited differing p53 responses: 50 µg/ml yielded 0.923±0.0717, 100 µg/ml was 1.32±0.0473, 200 µg/ml reached 1.44±0.0306, 300 µg/ml came in at 1.57±0.0498, and 500 µg/ml peaked at 1.75±0.0669. Untreated HeLa cells registered baseline p53 expression at 0.853± 0.147. Cisplatin at the typical 9 µg/ml dose substantially raised p53 to 1.60 ± 0.0536 through its established prompting of DNA damage and p53 activation. CM produced a concentration-reliant hike in p53: 50 µg/ml achieved 1.08± 1.08, 100 µg/ml reached 1.32± 0.0473, 200 µg/ml attained 1.45± 0.0351, 300 µg/ml amounted to 1.58± 0.0521, and 500 µg/ml topped out at 1.78± 0.0606.). Figures 3(D, E and F indicate that CM can fine-tune p53 expression in HepG2 cells, trending towards incremental p53 at over 100 µg/ml. These findings point to CM effectively upregulating p53 levels in HepG2 and HeLa cells, with the most prominent effects at 500 µg/ml. The pronounced p53 elevation at higher CM concentrations hints at its capacity for instigating cell cycle arrest and apoptosis in cancer cells. Baseline p53 expression in untreated 3T3 cells registered at 1.65±0.0376 under standard conditions. Treatment with the standard 9 µg/ml Cisplatin dose substantially reduced p53 to 0.663±0.0590, reinforcing its role in provoking p53 degradation or impedance. In contrast, CM treatment did not notably modify p53 expression versus the untreated control: 50 µg/ml was 1.65±0.0376, 100 µg/ml reached 1.68±0.0173, 200 µg/ml came in at 1.68±0.0780, 300 µg/ml was 1.68±0.0751, and 500 µg/ml amounted to 1.66±0.0872. These results indicate *Chrysanthemum morifolium* Ramat. did not impact 3T3 cell p53 levels under the tested circumstances, implying no effect on p53-governed cellular processes in normal cell systems.

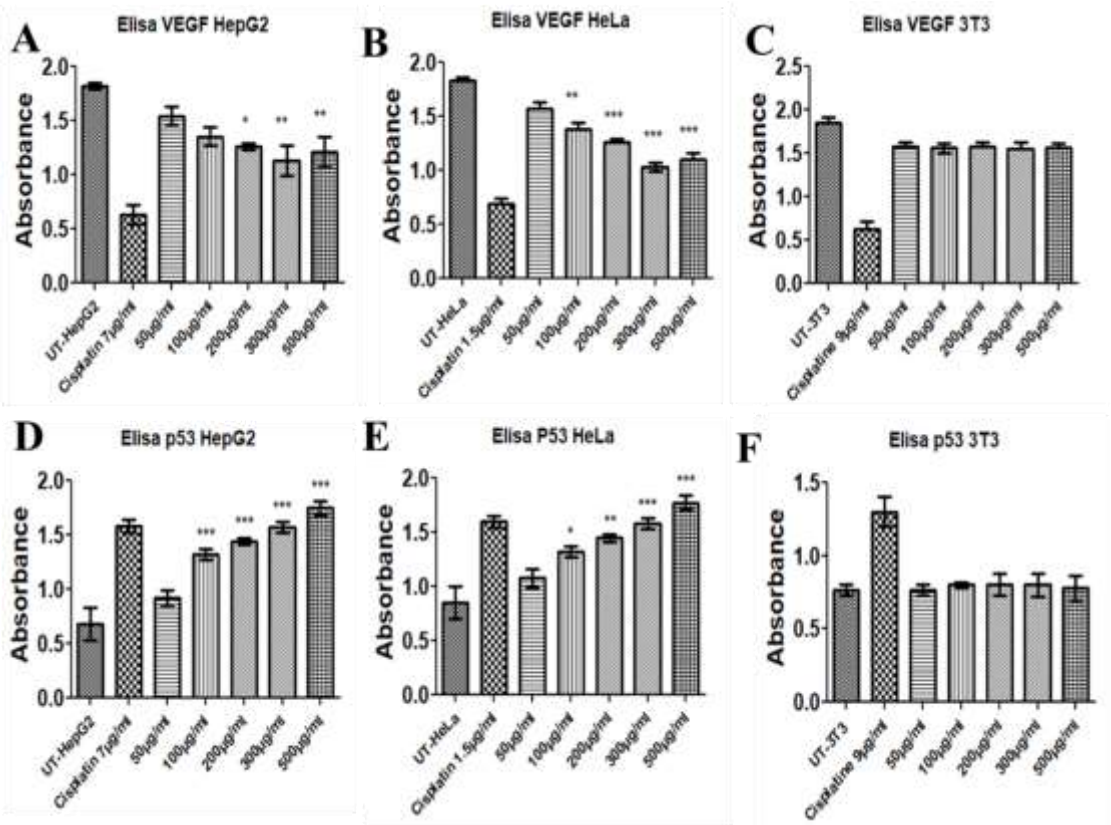


Figure 3: Reduced angiogenesis and enhanced apoptosis A) ELISA VEGF for HepG2. B) ELISA VEGF for HeLa. C) ELISA VEGF for 3T3. D) ELISA p53for HepG2. E) ELISA p53for HeLa. F) ELISA p53for 3T3. Absorbance values and percentage viability for untreated cells and the standard drug, Cisplatin were depicted. Values are expressed as mean ± SEM. Where $p<0.05$ is considered as significant.

Evaluation of antioxidant Assays

CAT ASSAY

The catalase activity assay was performed to evaluate the antioxidant enzyme activity in HeLa cancer cells treated with various concentrations of *Chrysanthemum morifolium* Ramat. (CM). Separately, the same assay was conducted to assess catalase activity in HepG2 cancer cells exposed to differing levels of CM. Untreated HepG2 cells exhibited an absorbance reading of 0.323±0.0467, signifying baseline catalase activity. By contrast, cisplatin, used as a standard drug at 7 µg/ml, significantly diminished catalase activity, resulting in an absorbance of 0.217±0.0348. However, CM treatment induced a concentration-dependent augmentation of catalase activity: 50 µg/ml (1.51±0.0404), 100 µg/ml (1.46±0.0404), 200 µg/ml (1.67±0.0318), 300 µg/ml (1.79±0.0416), and 500 µg/ml (1.97±0.0348). The absorbance value for untreated HeLa cells was 0.517±0.0384, representing the control catalase activity level. In sharp contrast, cisplatin at 1.5 µg/ml drastically reduced catalase activity, yielding an absorbance of 0.220±0.0361. Conversely, CM triggered a concentration-dependent surge in catalase activity: 50 µg/ml (1.51±0.0376), 100 µg/ml (1.40±0.0321), 200 µg/ml (1.66±0.0296), 300 µg/ml (1.79±0.0406), and 500 µg/ml (1.97±0.0273). The CAT assay sought to assess the enzyme activity in 3T3 normal cells following treatment with varying doses of CM. These data imply that CM enhances catalase activity in HepG2 and HeLa cells in a concentration-dependent fashion, with higher concentrations precipitating greater increases in enzyme function. The substantial elevation in catalase activity at 300 µg/ml and 500 µg/ml indicates CM may exert its anticancer effects through upregulating antioxidant defenses in HepG2 and HeLa cells. The absorbance value for untreated 3T3 cells was 0.553±0.0410, denoting baseline catalase activity. By contrast, cisplatin at 9 µg/ml dRamat.ically curbed catalase activity, yielding an absorbance of 0.287±0.0491. CM exposure induced a concentration-dependent rise in catalase activity: 50 µg/ml (1.68±0.0384), 100 µg/ml

(1.67±0.0367), 200 µg/ml (1.70±0.0491), 300 µg/ml (1.78±0.0441), and 500 µg/ml (1.82±0.0623) illustrated in figure 4(A). These findings imply CM enhances catalase activity in 3T3 cells in a dose-dependent manner, with higher concentrations precipitating greater increases in enzymatic function. The pronounced elevation in catalase activity at 300 µg/ml and 500 µg/ml indicates CM may reinforce antioxidant defenses in 3T3 cells.

APOX Assay

The ascorbate peroxidase assay was conducted to evaluate the enzymatic activity in HeLa cancer cells treated with varying amounts of *Chrysanthemum morifolium* Ramat. The absorbance reading for the untreated HepG2 cells was 0.350±0.0709, representing the baseline APOX activity level. Cisplatin, used as a standard drug at 7 µg/ml, substantially diminished APOX activity, yielding an absorbance of 1.43± 0.0555. In contrast, treatment with CM led to a concentration-dependent rise in APOX activity in a manner proportionate to the dosage: 50 µg/ml resulted in 0.543± 0.0677, 100 µg/ml produced 1.48±0.0380, 200 µg/ml caused 1.67±0.0338, 300 µg/ml induced 1.79±0.0406, and 500 µg/ml fostered 1.97±0.0273.

The absorbance value for untreated HeLa cells was 0.517±0.0384, representing the baseline level of APOX activity. Cisplatin, administered as a standard drug at 1.5 µg/ml, significantly curtailed APOX activity, yielding an absorbance of 1.44± 0.0694. Conversely, treatment with CM prompted a concentration-dependent surge in APOX activity proportional to the dosage: 50 µg/ml caused 0.557± 0.0731, 100 µg/ml induced 1.40± 0.0318, 200 µg/ml fostered 1.67±0.0338, 300 µg/ml elevated 1.79±0.0406, and 500 µg/ml heightened 1.97±0.0273.

These results demonstrate that CM substantially enhances APOX activity in HepG2 cells in a manner directly linked to concentration, with higher dosages producing more pronounced increases in enzymatic function. The marked elevation in APOX activity at 300 µg/ml and 500 µg/ml implies that *Chrysanthemum morifolium* Ramat.'s anticancer effects may stem from upregulating antioxidant defenses, especially APOX activity, in HepG2 and HeLa cells. This augmentation of APOX activity underscores the potential of CM to modulate oxidative stress responses, emphasizing its therapeutic promise for cancer treatment.

The baseline APOX activity level in untreated 3T3 cells was represented by an absorbance value of 1.28± 0.124. Treatment with Cisplatin, used as a standard drug at 9 µg/ml, significantly curtailed APOX activity to 1.45± 0.0608. In contrast, CM treatment prompted a conspicuous concentration-dependent rise in APOX activity proportionate to dosage: 50 µg/ml induced 1.32± 0.196, 100 µg/ml elevated 1.67±0.0367, 200 µg/ml heightened 1.70±0.0551, 300 µg/ml heightened 1.79±0.0406, and 500 µg/ml boosted 1.83±0.0669. These results suggest that CM substantially enhances APOX activity in 3T3 normal cells in a manner directly tied to concentration, with higher dosages inducing greater increases in enzymatic function per figure 4(B). The significant elevation in APOX activity observed at 300 µg/ml and 500 µg/ml implies that *Chrysanthemum morifolium* Ramat.'s effects may derive from augmenting antioxidant defenses, specifically by upregulating APOX activity, in 3T3 cells.

GSH Assay

The glutathione (GSH) assay was implemented to quantify intracellular GSH levels in HepG2 and HeLa cancer cells exposed to varying *Chrysanthemum morifolium* Ramat. (CM) concentrations. Untreated HepG2 cells displayed an absorbance of (0.553± 0.0745), representing baseline GSH. Cisplatin treatment at 7 µg/ml substantially decreased absorbance to (1.43± 0.0693), likely through cytotoxicity reducing GSH. Conversely, CM dose-dependently elevated GSH: 50 µg/ml (0.557± 0.0731), 100 µg/ml (1.57± 0.0410), 200 µg/ml (1.72± 0.0361), 300 µg/ml (1.76± 0.0644), 500 µg/ml (1.72± 0.0493). Untreated HeLa cells exhibited an absorbance of (0.557± 0.0731), denoting baseline GSH. Cisplatin at 1.5 µg/ml decreased absorbance to (1.44± 0.0694), reflecting reduced GSH. Conversely, CM dose-dependently raised GSH: 50 µg/ml (0.557± 0.0731), 100 µg/ml (1.58± 0.0406), 200 µg/ml (1.73± 0.0328), 300 µg/ml (1.76± 0.0669), 500 µg/ml (1.72± 0.0463). These findings imply CM enhances HepG2 cellular GSH, with highest at 500 µg/ml. The dose-response GSH increase suggests CM may boost antioxidant defenses by inducing GSH synthesis or diminishing depletion.

The potent GSH elevation even at lowest CM concentration underscores its powerful antioxidative effects. These results highlight CM's potential as a therapeutic to enhance cellular antioxidant capacity and battle oxidative stress in HepG2 cells. Untreated 3T3 cells displayed an absorbance of (1.26± 0.109), denoting baseline GSH. Cisplatin at 9 µg/ml significantly decreased absorbance to (1.42± 0.0549), likely indicating reduction due to Cisplatin-induced oxidative stress and toxicity. Conversely, CM substantially raised GSH across all concentrations tested: 50 µg/ml (1.31± 0.192), 100 µg/ml (1.68± 0.0328), 200 µg/ml (1.65± 0.0666), 300 µg/ml (1.80± 0.0379), 500 µg/ml (1.83± 0.0590). 3T3 cell GSH levels following CM treatment consistently surpassed untreated levels, with highest at 200 µg/ml CM per Figure 4(C). These results suggest CM enhances GSH in normal 3T3 cells, potentially conveying protection against oxidative damage. Elevated GSH across all CM-treated groups compared to untreated and Cisplatin-treated cells underscores CM's antioxidative properties. This supports CM's ability to bolster cellular antioxidant defenses, which could benefit reducing oxidative stress in normal cells.

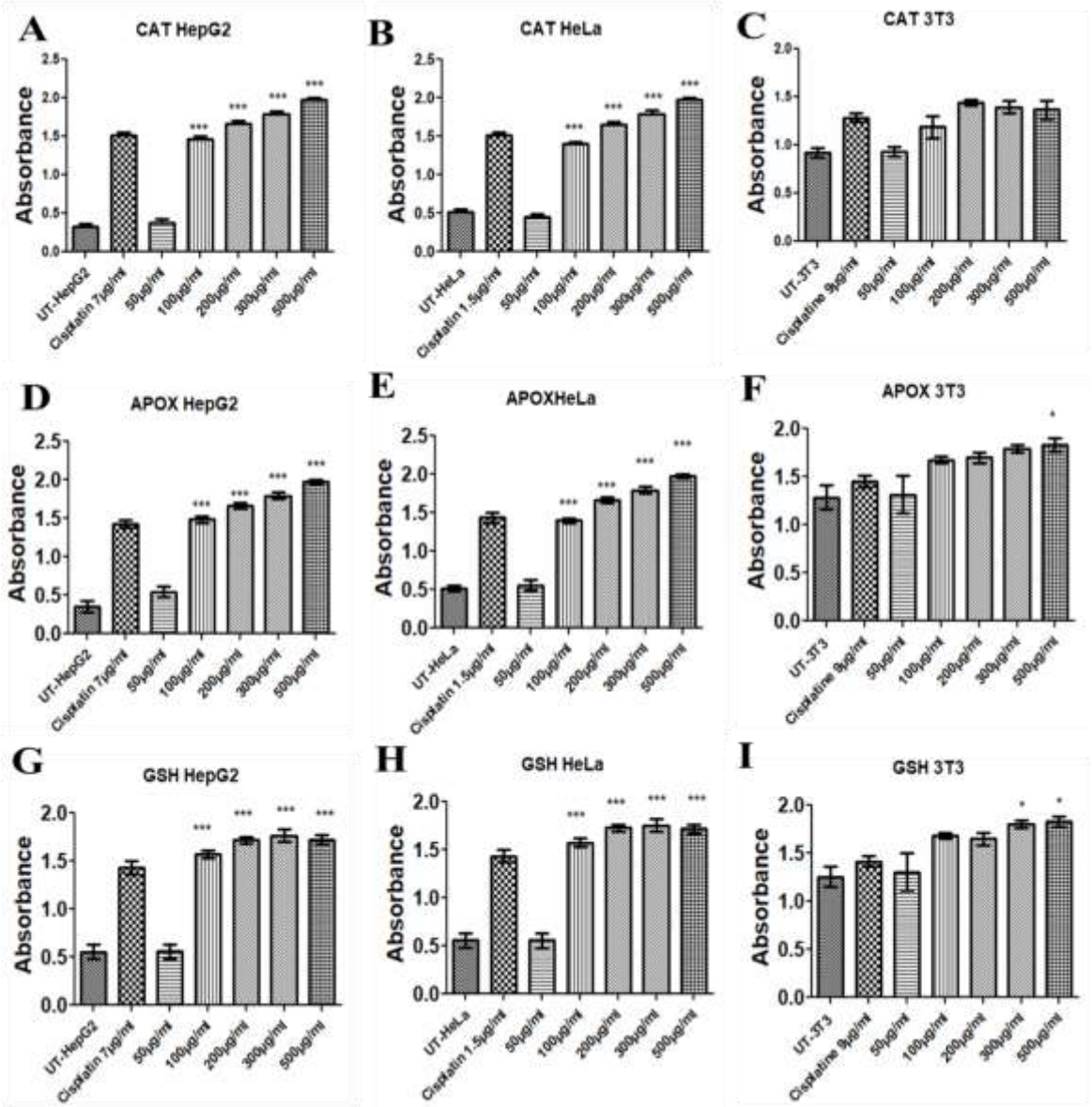


Figure 4: Evaluation of Antioxidant Potential of Chrysanthemum Extract. A) CAT assay for HepG2. B) CAT assay for HepG2. C) CAT assay for 3T3. D) APOX assay for HepG2. E) APOX assay for HeLa. F) APOX assay for 3T3. G) GSH asay for HepG2. H) GSH asay for HeLa. I) GSH assay for 3T3 Absorbance values and percentage viability for untreated cells and the standard drug, Cisplatin were depicted. Values are expressed as mean ± SEM. Where $p<0.05$ is considered as significant.

Discussion

The anticancer and antioxidant properties of *Chrysanthemum morifolium* Ramat. have been revealed through pharmacological investigation (Mohamad & Zahari, 2024). This study employed various assays to ascertain the cytotoxic and antioxidant effects of CM on HeLa cells (cervical cancer), HepG2 cells (liver cancer), as well as noncancerous 3T3 fibroblasts. The outcomes highlight CM's dose-dependent suppression of cancer cell viability while maintaining a nontoxic profile in normal cells, supporting its potential as a selective anticancer agent (Zhang et al., 2024).

The MTT assay results eloquently demonstrated CM significantly decreasing cell viability in HeLa and HepG2 cells in a concentration-dependent manner (Wageesha, Soysa, Atthanayake, Choudhary, & Ekanayake, 2017). At 500 µg/ml, CM reduced the absorbance values to a mere (0.263±0.111) for HeLa cells and a starkly meager (0.0666±0.0549) for HepG2 cells, signifying a powerful cytotoxic effect. The calculated IC50 values of 275 µg/ml for HeLa cells and 300 µg/ml for HepG2 cells further corroborate CM's efficacy at higher concentrations. In stark contrast, CM did not meaningfully impact the viability of 3T3 cells, with absorbance values staying comparable to untreated controls across all test concentrations. This implies CM exclusively targets cancer cells while sparing normal cells (Hodaei, Rahimmalek, & Behbahani, 2021).

The Crystal Violet and Trypan Blue assays corroborated the MTT assay findings, demonstrating a dose-dependent diminishment in cell viability and escalation in cellular demise in cancer cells exposed to CM. For the HeLa cells, the Crystal Violet assay revealed absorbance values declining from (0.317±0.0328) at 50 µg/ml to a strikingly low (0.0567±0.0176) at 500 µg/ml, highlighting CM's impressive influence. Correspondingly, the Trypan Blue assay indicated heightened cell death with rising CM treatment, with absorbance values rising markedly to (75.6±2.66) at 500 µg/ml for the HeLa cells. These conclusions underscore CM's potential to induce considerable cytotoxicity and cellular mortality in cancer cells in a potency dependent manner (K.-Y. Kim et al., 2019). The results of the LDH assay revealed that CM induces cytotoxicity by elevating the discharge of LDH in HeLa and HepG2 cells, proposing membrane harm and cell death (Xue et al., 2018). Furthermore, the ELISA assay demonstrated that CM upregulates p53 protein ranges, significantly at higher focus, signaling its position in selling apoptosis in most cancer cells (Gabr et al., 2022). As an instance, p53 ranges in HeLa cells elevated to (0.78±0.0606) at 500 µg/ml of CM, whereas HepG2 cells confirmed a related development. These discoveries propose that CM's anticancer results could also be mediated by means of the activation of apoptotic pathways (C. Kim et al., 2013). The examine additionally explored the antioxidant properties of CM utilizing catalase (CAT) and ascorbate peroxidase (APOX) assays. Each assay indicated that CM improves antioxidant enzyme exercise in most cancer cells. For instance, the CAT assay confirmed elevated exercise with absorbance values rising to (1.97±0.0273) at 500 µg/ml for each HeLa and HepG2 cells. Likewise, the APOX assay demonstrated elevated enzyme exercise at increased CM focus. These outcomes counsel that CM's anticancer results could also be partly resulting from its capacity to enhance the oxidative stress response in most cancers cells, thereby contributing to cell loss of life (Hadizadeh et al., 2022). Importantly, CM didn't exhibit cytotoxic results on 3T3 regular cells, as evidenced by constant absorbance values throughout all assays. This signifies that CM is non-toxic to regular cells and highlights its potential as a secure therapeutic agent (Damasco, Ravi, Perez, Hagaman, & Melancon, 2020). The ELISA assay confirmed minimal adjustments in VEGF ranges in 3T3 cells, suggesting that CM doesn't considerably affect angiogenesis in regular cells (Borgo, Laurella, Martini, Catalán, & Sülsen, 2021).

Conclusion

While the study demonstrates that *Chrysanthemum morifolium* Ramat. holds noteworthy anticancer properties, exemplified by prejudiced cytotoxicity, provoking of apoptosis, and improvement of antioxidant defenses in carcinoma cells, further probing is still needed. CM's absence of harm towards regular cells emphasizes its conceivable use as a healing operator. Long term examination should center around clarifying the sub-atomic instruments in charge of CM's impacts and assessing its viability in in vivo models to supplement approve its potential for clinical utilization in malignancy treatment. The findings unveil that CM acts selectively against cancer cells, killing them through

programmed cell death while protecting healthy tissues, pointing to promising avenues for more targeted therapies with reduced side effects. Nevertheless, more work must be done to fully comprehend exactly how CM exerts these anticancer activities on a molecular level before its therapeutic value can be confirmed in living systems.

Conflict of interest

The authors declare no conflict of interest.

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Authors' Consent

All contributors have critically reviewed and approved this manuscript for publication.

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