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IN VITRO STUDY TO EVALUATE THE ANTICANCER POTENTIAL OF *CHRYSANTHEMUM INDICUM*, THERAPEUTIC PLANT

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Abstract

Natural bioactive compounds that trigger apoptosis in medicinal plants might be a promising new source of anti-cancer medications. This study aimed to estimate the anti-cancer potential of the powerful medicinal plant *Chrysanthemum indicum*. The whole plant extract was extracted in *n*-hexane and ethanol solvents to test anticancer and antioxidative capabilities on the cancer HepG2 cell line. The cells in the untreated group had not been exposed to any of the plant extracts. Ethanol and *n*-hexane extracts were administered to the treated cell lines. Cell viability was estimated by employing standard methodologies for the anticancer potential. Cell death and viability were measured by MTT, crystal violet, and trypan blue tests. The rate of apoptosis was measured in the groups using an Annexin-V ELISA. Furthermore, antioxidant enzyme activity studies were carried out to investigate the scavenging activities of nitric oxide, DPHH radicals, H₂O₂, and superoxide, scavenging power of the extracts. Cell lines, HepG2 administered with plant extracts showed lower viability, decreased proliferation with increased apoptosis compared to untreated control cells, according to the findings. Plant extract-treated cancer cells have stronger anti-oxidative scavenging activity than untreated cancer cells. In comparison to the *n*-hexane extract, the ethanol extract exhibited stronger anti-cancer and antioxidative capability against cancer cells. It was concluded that *Chrysanthemum indicum* ethanol and *n*-hexane extracts induce anticancer activity in cancer cell lines by increasing apoptosis, antioxidant status, and decrease proliferation.

Keywords: Angiogenesis, Anticancer potential, Antioxidative activity, Anti-proliferation, apoptosis, *Chrysanthemum indicum*

INTRODUCTION

The growing global interest in medicinal plants indicates a growing acceptance of many traditional assertions about the benefits of natural products in health care. Plant medicines have a lower rate of adverse responses than current conventional pharmaceuticals, which, along with their cheaper cost, is prompting consumers and national health care organizations to explore them as alternatives to synthetic medications (1). Medicinal plants include essential phytochemicals with a wide range of pharmacological actions. Furthermore, derived beneficial compounds from therapeutic natural plants are frequently safer and less expensive than synthetically produced medications. As a result, there is a strong case to be made for natural plant-based medicines that are cost-efficient, safe, and effective (2). Secondary metabolites generated by plants are the source of numerous pharmacological drugs with antibacterial, hepatoprotective, anticancer, antiviral, anti-asthmatic, antipyretic and nephroprotective characteristics (3). Cancer is a disease characterized by uncontrolled cell propagation and reduced apoptosis that has a rapidly increasing global prevalence.

Over 3,000 plant species have been claimed to have cancer-curing properties, and thirty substances derived from these plants have been tested in cancer clinical trials (4). Due to their reduced toxicity and ease of accessibility equated to chemotherapy, these medicinal plant-imitative compounds have been utilized for



a long period as vital components of anti-cancer therapies which have played a significant role in cancer treatment. *Chrysanthemum indicum* L. (*C. indicum* L.), a perennial plant in the plant Compositae family, had been utilized as a customary medicine in China for about 2000 years (5) and was generally used as treatment for swelling, pemphigus, and scrofula. Above 190 biological compounds, including terpenoids, flavonoids, phenolic acids, and phenylpropanoids, have been identified and extracted from this plant so far (5). Extracted components from *C. indicum* L. have been proven in several recent research to exhibit anti-oxidant, anti-inflammatory, antipathogenic, anticancer, hepatoprotective, and immunological modulation properties (6). However, the mechanism research, resource availability, and quality control remain insufficient, necessitating more efforts.

The impact of *Chrysanthemum indicum* Linné extracts (CILE) on isoproterenol (ISO)-induced proliferation of human hepatocellular carcinoma (HCC) cells was examined in connection to MAPK/ERK1/2 intracellular activity. On both HepG2 and MHCC97H cells, CILE was found to be efficient in reducing the mitogenic impact of ISO. CILE's inhibitory impact was achieved in tumour cells by blocking the ISO-induced activation of MAPK/ERK1/2 through beta2-AR. Our findings will aid in the study of CILE's anticancer mechanism (7).

The study was therefore designed to investigate the anticancer potential of extracts of *Chrysanthemum indicum* in HepG2 cell lines. Our findings might be useful for future research because certain plant protective activities like antiproliferative potential of the whole plant's extract is the major point in focus of the current research. It would be desirable to do extensive scientific research and experiments on this remarkable medicinal herb to encourage its widespread use.

MATERIALS AND METHODS

EXTRACT PREPARATION

Chrysanthemum indicum plant was attained from the Biochemistry research lab of the University of Lahore's Institute of Molecular Biology and Biotechnology. The plant's components were rumped and processed to a powder using dry samples. The plant extract was extracted using the cold maceration procedure, which required putting a 400g plant sample in a container containing 1000ml of n-hexane and ethanol solvent in a shaker at room temperature for 7 days. After lyophilizing the extracts, stock solutions were prepared by dissolving 20 mg plant extracts in methanol (1 mL). The extract's yield (percent) was measured as follows:

$$\text{Yield (percentage)} = (w1 \times 100) / w2$$

w1 = Extract weight after solvent lyophilization w2 = Weight of powdered plant extract

SAMPLING OF CELL LINES

HepG2 (Human Liver Cancer) cell line and BHK (Baby hamster kidney) cell line were received from the cell culture lab of the university. The cell lines were kept alive in the cryovials holding liquid nitrogen. The cryovials were then resuscitated and administered in preparation for the subsequent step of the culturing process.

CULTURING CELL LINES

The cryovials had been defrosted after being frozen in liquid nitrogen. In a culture flask, HepG2 and BHK cell lines were cultivated in media (DMEM-HG) supplemented with 10% FBS, penicillin, and streptomycin. The culture was given to the growing cells when it had reached 70-80% confluency. The adhering cells were then rinsed in 1xPBS and treated with trypsin-EDTA until they were removed from the flask's surface. The detachment of cells was confirmed using an inverted microscope to examine the flask. FBS (a few drops) was added to the flask with constant shaking. After that, the contents of flask were centrifuged at 2000 rpm for five minutes. The supernatant was distant after centrifugation, trailed by the suspension of the pellet.

ADMINISTRATION OF PLANT EXTRACTS TO CELL LINES

For IC₅₀ measurement for muse analysis and cell viability, the HepG2 and BHK cells were grown in 96 well-microplates. Each cell line was splitted into three groups. One group of each received no therapy and was labeled UT-HG (untreated HepG2) and UT-BHK (untreated BHK). T-HG-CE (*C.indicum* ethanolic extract administered HepG2 cells) and T-HG-CH (*C.indicum* n-hexane extract administered HepG2 cells) were the two surviving cell line groups. There were two groups of control cell lines also which were designated as T-BHK-CE (*C.indicum* ethanol extract administered BHK cells) and T-BHK-CH (*C.indicum* n-hexane extract administered BHK cells). In DMEM, plant extract was given to each cell line at doses of (10,25, 50,100,200ug/mL).

ASSAYS FOR DETERMINING THE VIABILITY OF CELLS

ASSAY FOR MTT (FOR IC₅₀ CALCULATION)

The MTT test was performed according to a pre-determined protocol [16]. Plant extracts were given to HepG2 and BHK cells at doses of 10, 20, 50,100, and 200 ug/mL for 72 hours in a 96-well plate using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The cells' monolayer was rinsed in PBS (phosphate-buffered saline) buffer after 72 hours. Control samples were grown for 2 hours in a medium (100L complete medium containing 25 liters of MTT solution (without extracts). Tetrazolium in live cells converts to a purple color compound known as formazan which was solubilized subsequently in DMSO, and OD was taken at 570 nm.

TEST FOR TRYPAN BLUE (FOR DEAD CELLS DETECTION)

Trypan blue was used to measure cell viability since it inhibits both live and dead cells. Each experimental group's cells were rinsed three times in PBS before being treated in trypan blue for fifteen minutes (Invitrogen Inc., USA). Before being examined under the microscope, the cells were washed three times in PBS. It was supposed that the cells stained with trypan blue were dead. The following formula was used to determine the percentage of viable cells:

Viable cell percentage = $1.00 - (\text{Number of cells in blue color} / \text{total cell numbers}) \times 100$

Calculation of viable cells/ml after factoring in the dilution factor

$$\text{Number of viable cells} \times 10^4 \times 1.1 = \text{cells/mL culture}$$

ASSAY FOR CRYSTAL VIOLET STAINING (FOR LIVE CELLS ESTIMATION)

The viability of BHK and HepG2 cells was determined using crystal violet staining. 96-well plates were used for the experiment. After removing the secretome from the plate wells, the cells were washed with PBS. After washing, the wells were smeared with a 0.1 percent crystal violet and 2% ethanol combination that covered the whole surface. It was incubated then at room temperature for fifteen minutes. The dye was removed appropriately and the wells were cleaned thoroughly. After that, the stain was solubilized by adding 1 percent SDS to each well and letting it sit for 5 to 10 minutes. At 595nm, the absorbance was finally measured.

MUSE ANALYSIS WITH ANNEXIN V KIT

The Annexin V kit (Cat. No MCH100105) (Merck-Millipore) was used to treat HepG2 cells cultivated in plates (6 wells) with ethanol and n-hexane extracts of the plants at respective IC₅₀ concentrations using the Muse™ (Merck-Millipore) automated cell analyzer. After being post-treated, the cells were centrifuged at 2000rpm for 5 minutes at 2000 rpm. After 20 minutes of pellet desolation in Annexin v reagent, the supernatant was removed and the cells were counted. The Muse™ cell automated analyzer was used to determine the IC₅₀ value of plant extracts on HepG2 cells cultivated on plates (6 wells) using the Annexin V kit (Cat. No MCH100105) (Merck-Millipore).



ANTIOXIDANT POTENTIAL OF PLANT EXTRACTS ESTIMATION

ASSAY FOR DETERMINING THE DPPH POTENTIAL

In 99.5 percent methanol, a 1mM 2-diphenyl-1-picryl-hydroxyl solution was dissolved. The standards (1ml sample having 50 ppm or 10 mg/ml concentration) of Butyl hydroxyl anisole were mixed with a 250 mL DPPH solution and incubated for thirty minutes in the dark. The absorbance readings were taken at 517nm using SpectraMax 190 Microplate reader. As a blank, methanol was used. The following formula was used to compute the fraction of DPPH free radical inhibition:

$(\text{Abs of blank} - \text{Abs of the test sample}) / (\text{Abs of blank}) \times 100 = \text{Inhibition (percentage)}$

NITRIC OXIDE (NO) SCAVENGING POTENTIAL ESTIMATION

The scavenging potential was determined using the approach given by (8).

SCAVENGING POTENTIAL OF SUPEROXIDE ANIONS ESTIMATION

The scavenging potential was determined using the approach given by (9).

HYDROGEN PEROXIDE (H₂O₂) SCAVENGING POTENTIAL ESTIMATION

The potential for H₂O₂ scavenging was determined using the approach outlined by (10).

ESTIMATION OF THE POTENTIAL FOR REDUCING POWER

The approach used to determine Reducing Power Potential was shown by (9).

ANALYTICAL STATISTICS

The mean SEM of the results obtained from, cell viability tests, and antioxidative potential assessments of trial groups were used. At the significance level, a one-way ANOVA statistical test was employed for the group means comparison ($p < 0.05$). The entire data was analyzed by using Graph pad prism (9.1.2 software).

RESULTS

Ethanol and n-hexane extracts of *Chrysanthemum indicum* were tested in vitro for antiproliferative, apoptotic, and cytotoxic effects as well as antioxidative properties utilizing HepG2 and BHK cell lines. For cytotoxicity, antiproliferative, apoptotic, and antioxidative potential measurement, MTT, trypan blue, and crystal violet assay along with nitric oxide, H₂O₂, superoxide, and DPPH scavenging activity assessments were conducted and the outcomes were compiled as follows.

ASSESSMENT OF CYTOTOXIC EFFECT OF CHRYSANTHEMUM INDICUM EXTRACTS ON HEPG2 CELLS

The MTT test was discovered to be a consistent and simple technique to measure cell cytotoxicity. Fig. 1. Demonstrates the cytotoxicity of *Chrysanthemum indicum*, ethanol, and n-hexane extracts as % sustainability of cells vs. concentrations (10, 25, 50, 100, 200ug/ml). At the highest concentrations, both the extracts of the plant exhibited a dose-dependent mechanism of cytotoxicity.

The IC₅₀ values for both plant extracts are summarized in Table I. The IC₅₀ values for *C. indicum* extract in ethanol and n-hexane were found to be 86.2±21.8ug/ml and 83.06±19.2ug/ml, respectively. These IC₅₀ values were employed in the study's subsequent tests. Because the extract's inhibitory action is maximum at even lower doses, a lower IC₂ value suggests higher cytotoxicity. The results showed that ethanol extract of *C. indicum* had a higher level of cytotoxicity (lower IC₅₀) than n-hexane extract, Significant difference ($F=111.2, p=0.001 < 0.05$) in cytotoxicity potential of ethanol and n-hexane extracts were observed among both cell line groups (T-HG-CE, T-HG-CE).

Table I. Cytotoxicity level of *Chrysanthemum indicum* ethanol and n-hexane extracts

Groups (Cell Lines)	Samples	IC ₅₀ Values (±SEM) (ug/ml)	F	p- value
T-HG-CE	Ethanol extract administered HepG2	86.2±21.8		
T-HG-CH	n-hexane extract administered HepG2	83.06±19.2	111.2	0.0001*

*Significant value p<0.05

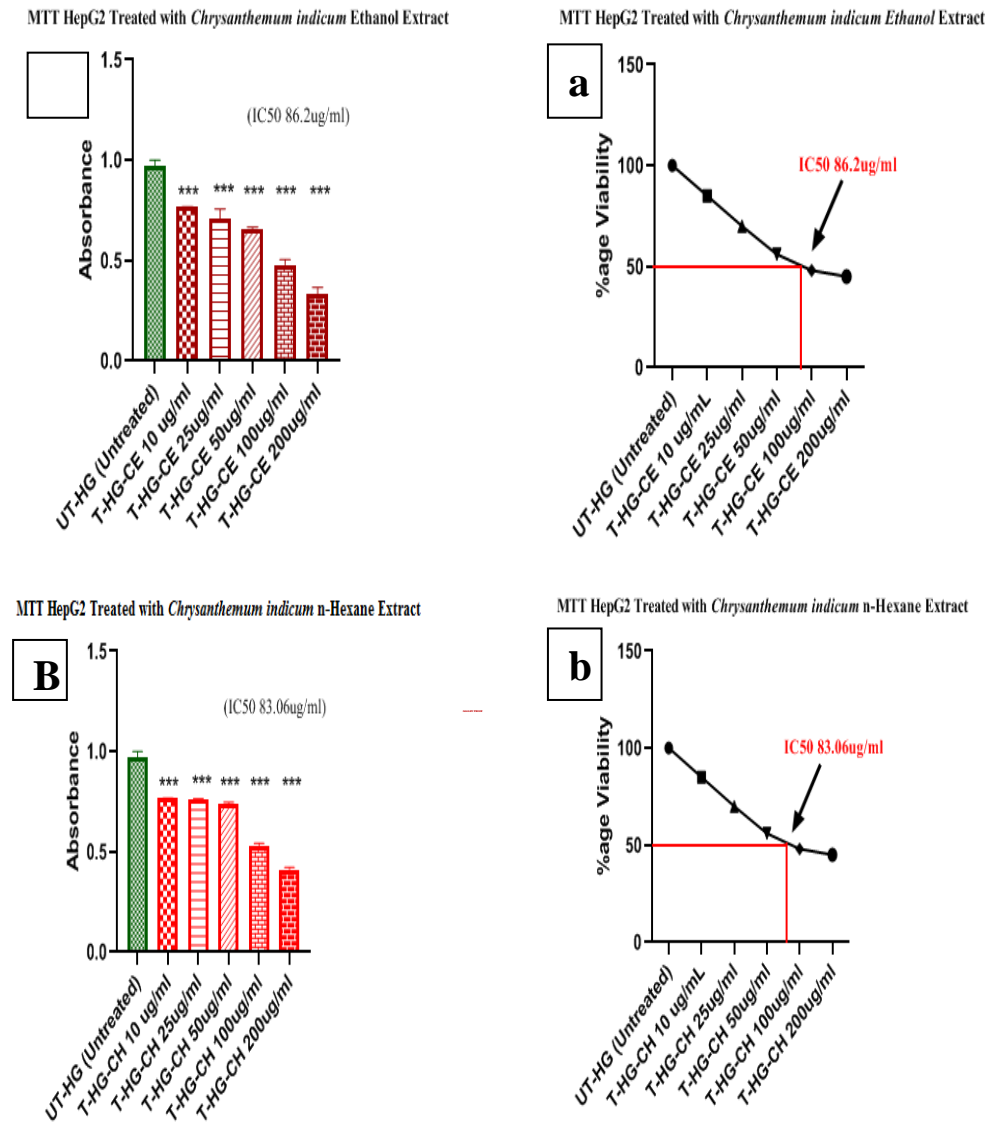


Fig. 1. MTT test to estimate cytotoxicity at IC₅₀ values of extracts of *C. indicum* on HepG2 cells, (A) Cytotoxicity induced at different *C. indicum* ethanol extract concentrations in HEPG2 cells. (a) IC₅₀ values of different concentrations of *C. indicum* ethanol extract. (B) Cytotoxicity induced at different *C. indicum* n-hexane extract concentrations in HepG2 cells (b) IC₅₀ values of different concentrations of *C. indicum* n-hexane extract

ESTIMATION OF EFFECT OF *CHRYSANTHEMUM INDICUM* EXTRACTS ON CELL VIABILITY

The impact of plant extracts on HepG2 cell survival was investigated by treating the cells with each extract at its IC₅₀ gained from the MTT experiment. Crystal violet and trypan blue tests were used to measure cell viability.

TRYPAN BLUE ASSAY

In comparison to untreated HepG2 cells, treated HepG2 cells had a considerably larger number of dead cells (F=438.3, p=0.0001<0.05) (Fig. 2A). The mean dead cell (percentage) observed in the UT-HG was 10.67±1.155 while the mean dead cells percentage observed in the treated groups T-HG-CE and T-HG-CH respectively were 41.67±2.082 and 47.33±1.528 (Table II).

There were fewer dead cells in BHK normal cell lines than in HepG2 treated cells (Figure 2B), indicating that *C. indicum* extracts had no antagonistic effects on normal cells. The mean dead cells percentage in an untreated UT-BHK was 15 ± 1.000 whereas it was 15.50 ± 1.000 and 15.33 ± 1.258 in the T-BHK-CE: T-BHK-CH: BHK groups treated with *C. indicum* ethanol and n-hexane extracts respectively (Table II). Insignificant statistical difference was observed in mean dead cells in the untreated and treated BHK cell line ($F=0.1628$, $p=0.8534 > 0.05$).

Table II. Trypan blue assay for the estimation of cell viability

Groups (HepG2 cell lines)	Dead cell (%) values (\pm SEM)	F	p-Value	Groups (BHK cell lines)	Dead cell (\pm SEM) (%) values	F	p-Value
UT-HG	10.67 ± 1.155			UT-BHK	15 ± 1.000		
T-HG-CE	41.67 ± 2.082	438.3	0.0001*	T-BHK-CE	15.50 ± 1.000	0.1628	0.8534
T-HG-CH	47.33 ± 1.528			T-BHK-CH	15.33 ± 1.258		

*Significant value $p < 0.05$

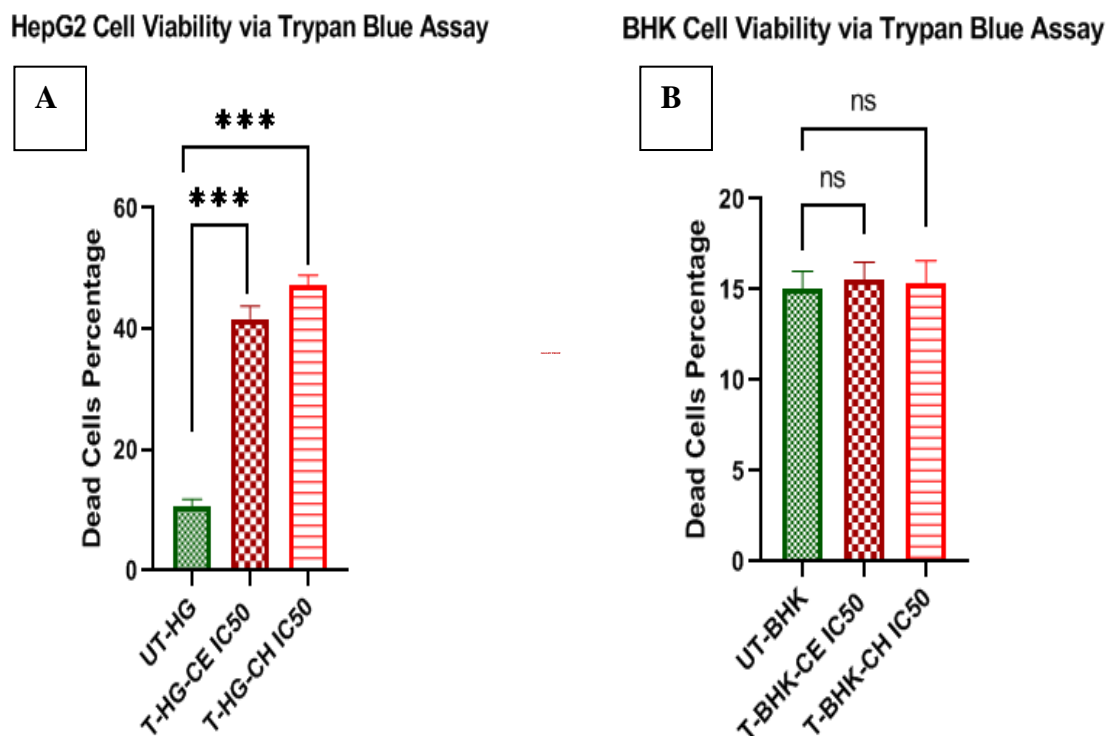


Fig. 2. Estimation of dead cells percentage under trypan blue test, (A) HepG2 Cells viability (%) (B) BHK cells viability (%)

ASSESSMENT OF % CELL VIABILITY UNDER CRYSTAL VIOLET STAINING

The viability of HepG2 cells treated with plant extracts was measured by employing crystal violet staining, which revealed a significantly reduced absorbance (greater number of dead cells). In the untreated HepG2 cell line, the mean absorbance linked to dead cells was 0.8667 ± 0.07638 , but the mean absorbance in the groups treated with ethanol and n-hexane extract was 0.4167 ± 0.07638 and 0.4333 ± 0.08505 , respectively. In comparison to untreated HepG2 cells, treated HepG2 cells had a considerably larger number of dead cells ($F=31.00$, $p=0.0007 < 0.05$) (Fig. 3A).

The high absorbance values in BHK cell lines correspond to fewer dead demonstrating apoptotic action of the extracts. The mean absorbance values in the treated and untreated BHK cell line groups were insignificant statistically ($F=0.005118$, $p=0.9949 > 0.05$) (Table III, Fig. 3B).

Table III. Assessment of % Cell Viability under Crystal Violet Staining

Groups (HepG2 cell lines)	Absorbance (±SEM)	F	p-Value	Groups (BHK cell lines)	Absorbance (±SEM)	F	p-Value
UT-HG	0.8667±0.07638			UT-BHK	1.033±0.5208		
T-HG-CE	0.4167±0.07638			T-BHK-CE	1.000±0.5000		
T-HG-CH	0.4333±0.08505	31.00	0.0007*	T-BHK-CH	1.000±0.3606	0.005118	0.9949

*Significant value p<0.05

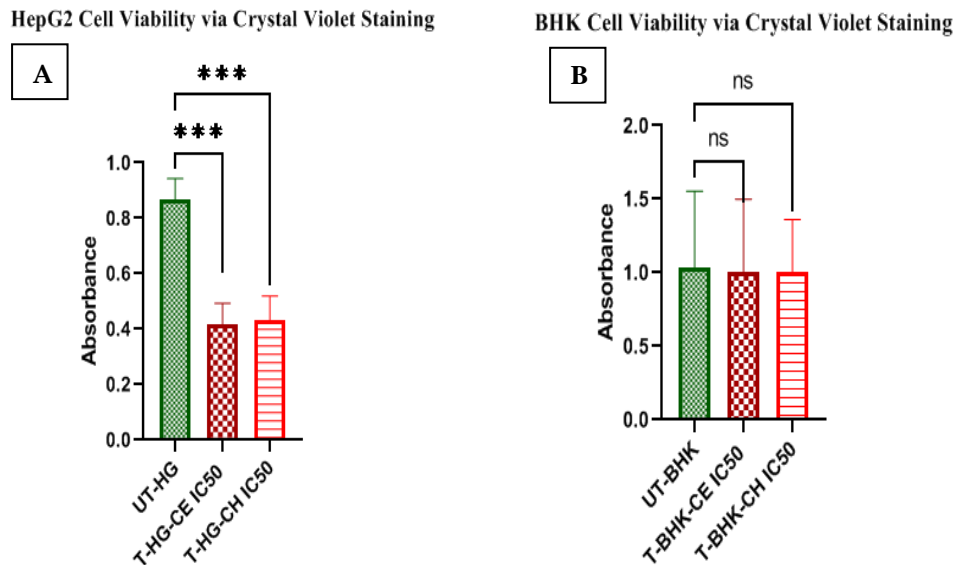


Fig. 3. Estimation of dead cells percentage under crystal violet staining (A) HepG2 Cells viability (%) (B) BHK cells viability (%)

ESTIMATION OF APOPTOTIC POTENTIAL OF *CHRYSANTHEMUM INDICUM* PLANT EXTRACTS

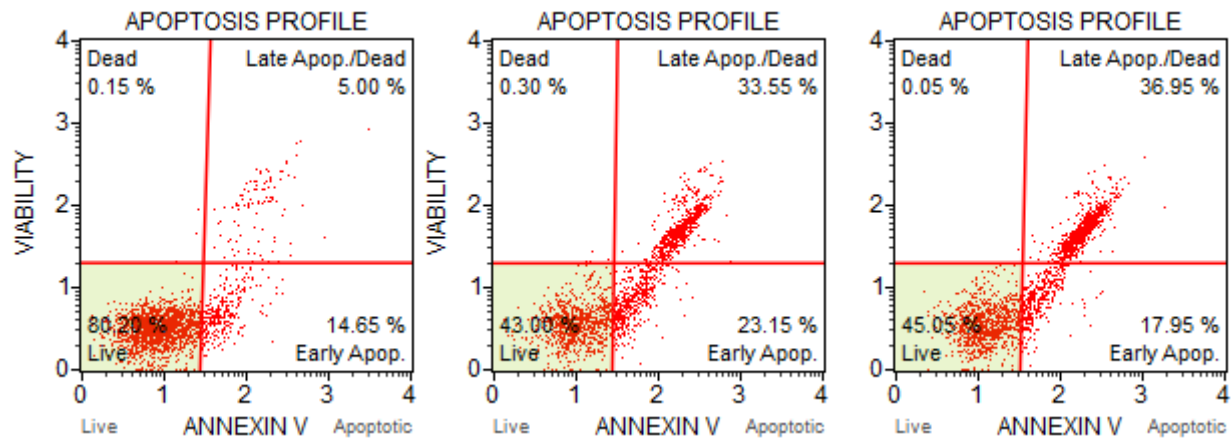
The degree of apoptosis was determined using the kit's annexin-V reagent. It was observed that the treated groups, T-HG-CE and T-HG-CH, showed a considerably higher proportion of apoptosis (late) and hence more dead cells. In the untreated UT-HG group, the proportion of first living cells was 80.20 percent. After incubation time, the percentage of live cells was 80.20 percent, the % late apoptotic cells were 5.00 percent, and the percentage of dead cells was 0.15 percent (Fig. 4A). The T-HG-CE group had 45.05±.42 percent live cells, 17.95±0.37 percent early apoptotic, 36.95±0.38 percent late apoptotic cells (Figure 5B). % live cells for the T-HG-CH group was recorded as 43±0.32 with early apoptosis 23.15±0.43%, late apoptosis 33.55±0.13%. (Fig. 5C). Significantly elevated (F=1021, p=0.0001<0.05) late apoptosis percentage was obtained in both the treated groups as compared to the untreated group. It was also explored from the results that the percentage of late apoptosis was high in group treated with ethanol extract as compared to the n-hexane administered group of HepG2 cells however the difference in mean values was statistically insignificant (F=2111, p=0.613>0.05) (F=1231, p=0.50>0.05) (Table IV).

Table IV. Assessment of Apoptotic Activity for HepG2 Cells

Groups (HepG2 cell lines)	Live cells %±SEM	Early Apoptosis %±SEM	Late apoptosis %±SEM	F	p-value
UT-HG	80.20±0.011	14.65±0.056	5.00±0.022	1021	0.0001*
T-HG-CE	45.05±.42	17.95±0.37	36.95±0.38	2111	0.613
T-HG-CH	43±0.32	23.15±0.43	33.55±0.13	1231	0.50

*Significant value p<0.05





(A) UT-HG

(B) T-HG-CE

(C) T-HG-CH

Fig. 4. Assessment of apoptotic potential of *C.indicum* plant extracts. (A) Apoptosis in UT-HG. (B) Apoptosis in T-HG-CE group after *C.indicum* ethanol extract administration. (C) Apoptosis in T-HG-CH group after *C.indicum* n-hexane extract administration

IN VITRO ASSESSMENT OF ANTIOXIDANT ACTIVITY OF *CHRYSANTHEMUM INDICUM* EXTRACTS

The antioxidative potential of *Chrysanthemum indicum* plant extracts was evaluated using H₂O₂, superoxide, nitric oxide, and DPPH scavenging activity (SA) assays. The results showed that the treated T-HG-CE and T-HG-CH groups had significantly higher H₂O₂ (SA) (F=111.1, p=0.0001<0.05) (Fig. 5A), nitric oxide (SA) (F=74.45, p=0.0001<0.05) (Figure 5B), superoxide (SA) (F=1260, p=0.0001<0.05) (Fig. 5C), and DPPH radical (SA) (F=161.7, p=0.0001<0.05) (Fig. 5D). The antioxidative activities of treated and untreated HepG2 cell lines were found to be differing significantly. There was no significant difference in H₂O₂ (SA) (F=2.739, p=0.1428>0.05) (Fig. 5A), nitric oxide (SA) (F=3.000, p=0.1672>0.05) (Fig. 5B), superoxide (SA) (0.2568, p=0.7816>0.05) (Fig. 5C), and DPPH radical (SA) (F= 0.02801, p=0.9725) (Fig. 5D). Overall insignificant statistical difference in the antioxidant levels was detected in untreated and treated BHK normal cell lines (Table V).

Table V. Assessment of antioxidant activity of *Chrysanthemum indicum* extracts

Antioxidant activity	Groups (HEPG2)	Absorbance (±SEM)	F	p-Value	Groups (BHK)	Absorbance (±SEM)	F	p-Value
H ₂ O ₂ (SA)	UT-HG	0.1155±0.005500	111.1	0.0001*	UT-BHK	0.3233±0.01528	2.739	0.1428
	T-HG-CE	0.3347±0.04008			T-BHK-CE	0.3300±0.01000		
	T-HG-CH	0.3860±0.006000			T-BHK-CH	0.3500±0.01000		
Nitric oxide scavenging activity	UT-HG	0.3333±0.05774	74.45	0.0001*	UT-BHK	0.3233±0.01528	3.000	0.1672
	T-HG-CE	0.6900±0.03606			T-BHK-CE	0.3300±0.01000		
	T-HG-CH	0.7700±0.04359			T-BHK-CH	0.3500±0.01000		
Super oxide scavenging activity	UT-HG	0.3500±0.03000	1260	0.0001*	UT-BHK	0.3500±0.03000	0.2568	0.7816
	T-HG-CE	2.723±0.1079			T-BHK-CE	0.3433±0.03055		
	T-HG-CH	2.867±0.04163			T-BHK-CH	0.3333±0.02517		
DPPH radical scavenging activity	UT-HG	0.1155±0.005500	161.7	0.0001*	UT-BHK	0.3000±0.1000	0.02801	0.9725
	T-HG-CE	0.4451±0.03046			T-BHK-CE	0.3073±0.08456		
	T-HG-CH	0.5000±0.1000			T-BHK-CH	0.3177±0.09036		

*Significant value p<0.05



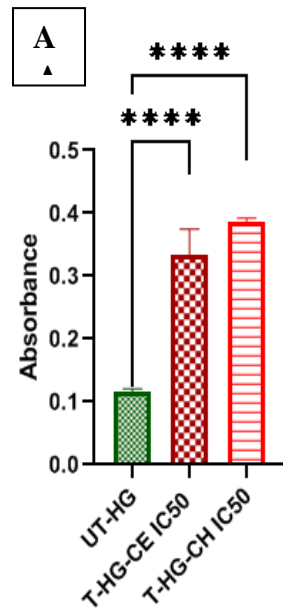
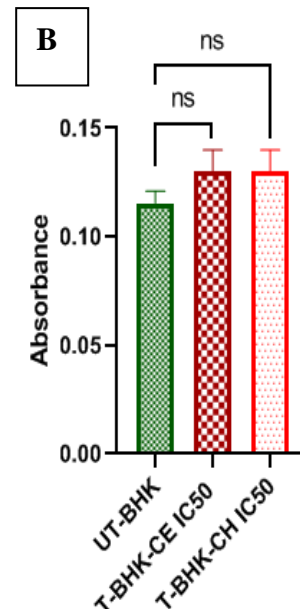
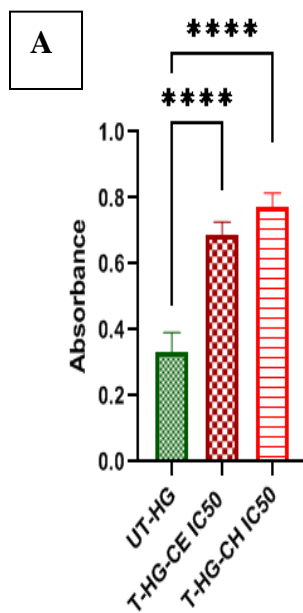
H₂O₂ Scavenging Activity in HepG2 CellsH₂O₂ Scavenging Activity in BHK Cells

Fig. 5A. H₂O₂ scavenging activity of *C.indicum* extract in ethanol and n-hexane (A) H₂O₂ (SA) in HepG2 cell line (B) H₂O₂ (SA) in BHK cell line

Nitric oxide Scavenging Activity In HepG2 Cells



Nitric oxide Scavenging Activity In BHK Cells

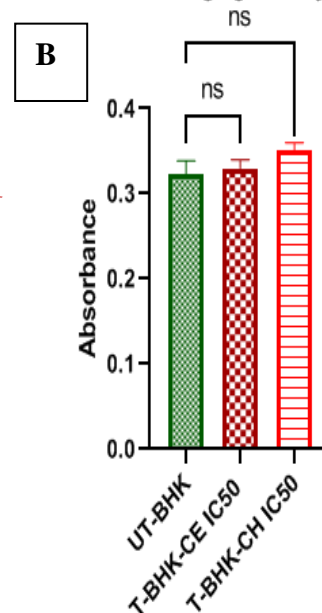


Fig. 5B. Evaluation of nitric oxide scavenging activity of *C.indicum* extract in ethanol and n-hexane, (A) Nitric oxide (SA) in HepG2 cell line, (B) Nitric oxide (SA) in BHK cell line

DISCUSSION

Natural medicines, such as phytopharmaceuticals yields and phytotherapeutic agents, are regarded to be safer than manufactured medicines and are drawing human attention (11). This has rekindled scientists' interest to determine the medicinal properties of natural products. To discourse the problem of treatment management resistance in various malignancies, researchers are working on novel anti-tumorigenic compounds that have a powerful anti-cancerous influence on cancer cell development (12). Even though certain drugs are low in toxicity, inhibit the angiogenic ability of cancer cells (13). At the moment, naturally occurring chemicals, particularly those originating from medicinal plants, are viewed as critical players in the development of novel medications for life-intimidating disorders. Furthermore, research has demonstrated that antioxidant-rich phytotherapeutic medicines may lessen the incidence of cancer (14). So, new anti-cancer medicines derived from plants with substantial antioxidant activity are urgently needed.

The current study examined the apoptotic and cytotoxic effects, as well as the antioxidative potential, of ethanol and n-hexane extracts of *C.indicum* plant in vitro using HepG2 cell lines as cancerous and BHK cells as normal control cell lines. To assess the cytotoxicity of plant extracts, the MTT assay was utilized. MTT is a tetrazolium salt (positively charged) that is rapidly reduced by the cells carrying active metabolism, resulting in formazan formation (purple-colored). The MTT test is used to evaluate cell viability due to its reductive activity (12). Our findings showed that HepG2 cells were considerably reduced in their proliferation when treated with both ethanol and n-hexane *C.indicum* extracts and that untreated HepG2 cell lines had less number of dead cells than the cells of treated group. The proliferation of BHK cells in treated and untreated groups remains unaffected. Recent research (15) found that a pro-apoptotic protein (15-kDa) from *C.indicum* stimulates caspase-3 while down regulating Bcl-2 gene expression in murine cancer cells, indicating apoptotic activity of the plant extract. Additional the other cell sustainability tests, such as crystal violet and trypan blue, and muse analysis, also produced comparable results, with the high number of dead cells in *C.Indicum* extract administered HepG2 than BHK cell lines. Chrysanthemum indicum extract suppresses MAPK and NF-kappaB activity in lipopolysaccharide-tempted RAW 264.7 which inhibits the inflammatory response in liver cancer (16).

We also investigated plants for antioxidant capacity using DPPH, nitric oxide, superoxide, hydrogen peroxide (H₂O₂) scavenging activity, because oxidative stress is a key marker for cancer (17). Previous research (4, 13, 15, 18) has shown that combining antioxidants with anti-proliferative medicines enhances the therapeutic efficacy and increases anti-cancer effects. Oxidative stress generates an increased reactive oxygen species (ROS), that is used by the cancer cells by metabolic shift for the protection from oxidative damage-induced apoptosis, allowing for fast growth.

It was perceived that in extracts administered HepG2 cells, the antioxidative activities enhanced, that lower the oxidative stress and improve anti-proliferative efficiency as compared to untreated HepG2 and BHK cells. Overall, the current study discovered that the plant under review had anti-proliferative, apoptotic, and antioxidative characteristics against cancer. It was also observed that the ethanol extracts of the plant offer most effective anti-cancer therapeutic potential than the n-hexane extract. It was also shown that ethanol extract of the plant had a higher apoptotic and antioxidative potential than n-hexane extracts. This might be due to the ethanol extract of the plants having a higher production of total flavonoids and total phenols, which are the most effective anti-cancer agents.

The majority of plant derived anticancer medications work by causing tumor cells to die. The majority of plant-derived anticancer medicines induce intrinsic or extrinsic apoptosis, as well as caspase and/or p53-dependent or independent processes (19). Annexin V staining reveals a substantial proportion of apoptosis after treatment with plant extracts in multiple studies (6, 20). The plants' ethanol and hexane induces increased apoptotic activity in HepG2 cells through annexin V-dependent pathways, resulting in a more apoptotic activity in the cells. Overall, the current investigation discovered that the plants' ethanol and n-hexane extracts had apoptotic, cytotoxic, and antioxidative properties against the cancer cell line HepG2.

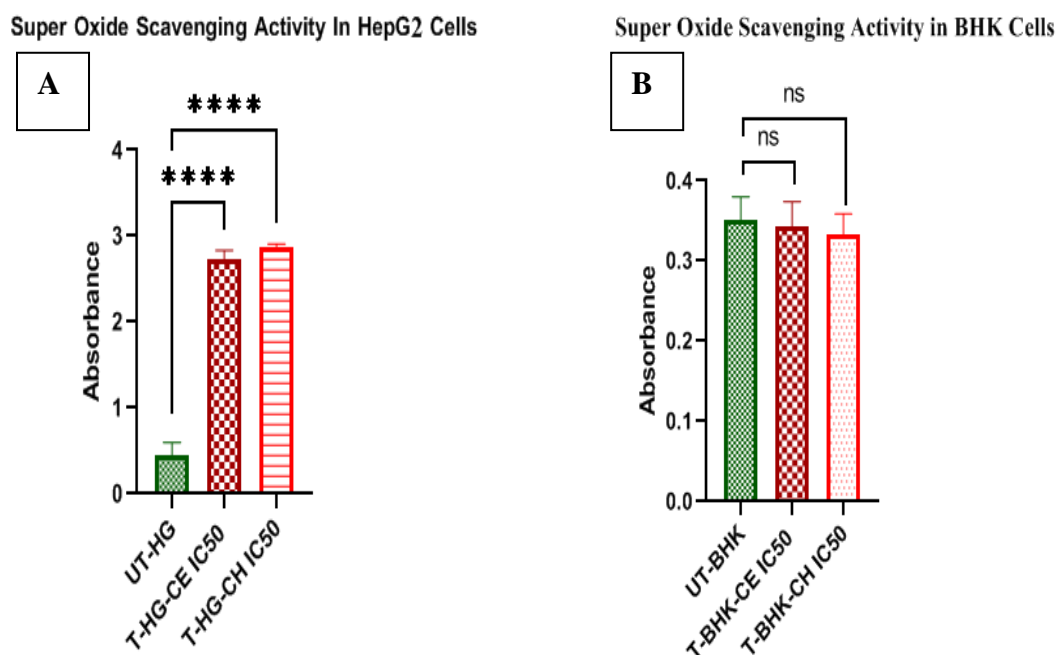
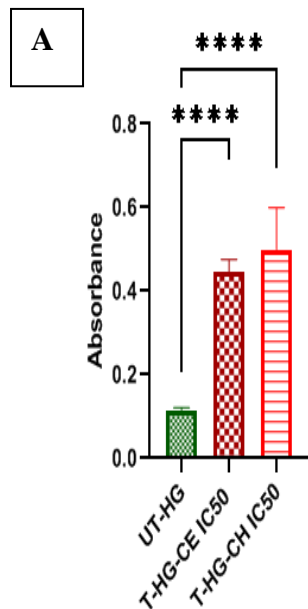


Fig. 5C. Evaluation of superoxide scavenging activity of *C. indicum* extract in ethanol and n-hexane, (A) Superoxide (SA) in HepG2 cell line (B) Superoxide (SA) in BHK cell line.

DPPH Radical Scavenging Activity In HepG2 Cells



DPPH Radical Scavenging Activity in BHK Cells

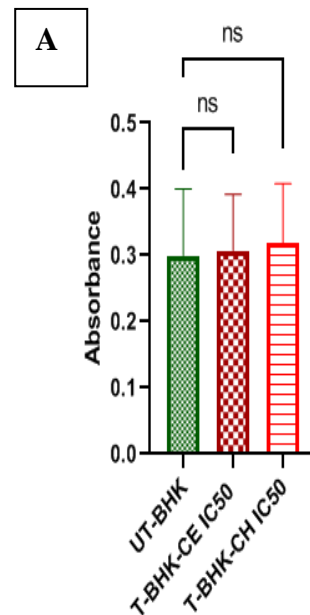


Fig. 5D. Evaluation of DPPH scavenging activity of *C.indicum* extract in ethanol and n-hexane, (A) DPPH radicals (SA) in HepG2 cell line, (B) DPPH radicals (SA) in BHK cell line

CONCLUSION

The ethanol and n-hexane extracts of three therapeutic plants can trigger apoptosis in cancer cell lines (HepG2) and inhibit their proliferation while also increasing their antioxidant capacity. It was inferred from the present investigation that the n-hexane and ethanol extracts of *C. indicum* extracts can prompt apoptosis, cytotoxicity, and antioxidant activity in cancer cells to reduce the growth of cancer cells. The findings also demonstrated that ethanol has a more effective anticancer potential than the n-hexane extract of *C. indicum*.

Conflict of Interest

The authors declare no conflict of interest.

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