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Evaluation of the cytotoxicity of *Alpinia officinarum* rhizome extract against some cancer cells in vitro

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Summary

The current study aimed to evaluate the cytotoxicity of aqueous and alcoholic extracts of galangal rhizome against certain cancer cells and to select the optimal cell line exhibiting the highest inhibition rate, the chemical content and total phenolic and flavonoid content were estimated, and antioxidant levels were measured using DPPH assay, the cytotoxicity of the galangal extracts was then investigated using the MTT assay to determine their toxicity and ability to inhibit the growth of specific cancer cells, namely HepG2 liver cancer cells, Coco-2 colon cancer cells, and MCF-7 breast cancer cells, and compared to normal WRL68 cells in vitro. The results showed a variation in the chemical content of galangal rhizome, and also showed a significant difference in the amount of flavonoids and phenols between the extracts. Compounds effective against microorganisms and cancer were obtained using GC-Mass, and there were significant differences in antioxidants at concentrations of 0.12 and 0.25 mg/ml, and no significant differences at concentrations of 0.5 and 1 mg/ml between the aqueous and alcoholic extracts. The results also showed that the aqueous and alcoholic extract of galangal rhizome effectively inhibits the growth of cancer cells at a concentration of 400 µg/mL, with inhibition rates of (68.29, 58.72, 35.61) % for the cell types respectively. The HepG2 liver cancer cell line was selected based on the MTT results, as it had the highest inhibition rate for the cancer cells under study. Cytotoxicity was then assessed by performing the HCS detection assay to measure the cellular indicators represented by Viable Cell Count (VCC), Total Nuclear Intensity (TNI), Cell Membrane Permeability (CMP), Mitochondrial Membrane Potential (MMP), and Cytochrome C releasing (CC). The results indicated a decrease in the number of viable cell carcinomas (VCCs) of HepG2 liver cells, particularly at concentrations of 100 and 200 µg/mL, by 34.48% and 38.22%, respectively. There was also a significant increase in (TNI) at a significance level of $p < 0.05$, by 26.85% and 66.88%, respectively. A clear effect on (MMP) was also observed, by 26.45% and 31.03%, respectively. Furthermore, there was a significant increase in cytochrome C (CC) release levels, by 47.67% and 62.63%, respectively. These findings suggest that the galangal rhizome extract possesses high inhibitory activity against the cancer cells under study in vitro.

Keywords: Antioxidant; phenols; Flavonoids; Cell line cancer; liver cancer HepG2

Introduction

Cancer is one of the most common diseases in which a group of cells grows and multiplies abnormally and uncontrollably, allowing them to continue multiplying and spreading throughout the body, these cells have the ability to invade and damage adjacent tissues, and then move between tissues, eventually reaching most of the surrounding and distant tissues (Al-Shaikh and Doosh, 2024; Johariya *et al*., 2024), this growth results in the massing and swelling of tissues, which are called tumors, these tumors are classified into two types: malignant tumors and benign tumors, benign tumors are non-cancerous tumors, but their growth is abnormal, they are not life-threatening because they do not spread and can be easily removed through surgical procedures, malignant tumors, on the other hand, are among the most dangerous tumors, characterized by their rapid proliferation and spread to all organs and adjacent tissues, causing weakness in their functions and then damage to them and spread to other tissues (Porrello *et al*., 2023).

Liver cancer is a cancer that begins in the cells of the liver, the liver is the largest organ in the human body, located under the right rib below the right lung of the body, and weighs approximately 1.2-1.4 kg, it plays a major role in metabolism and has a number of functions, including the production of bile and the storage of glycogen, the breakdown of red blood cells, the production of plasma protein, the manufacture of vitamins and hormones, and the removal of toxins from the blood and drug toxins, the liver is one of the organs that has the ability to regenerate, as well as many immune functions (Nieminen and Lemasters, 2023). Liver cancer is the second leading cause of cancer death among men in developed countries, after lung cancer (Torre *et al*., 2015). It is also the leading cause of cancer death worldwide, with most reports indicating that it causes approximately 600,000 deaths annually, the primary causes of liver cancer remain largely unknown (Zamio, 2024). Liver cancer has been found to be associated with congenital defects, chronic infections such as hepatitis B and C, hemochromatosis, and cirrhosis, as well as alcohol consumption. Identifying the different subtypes of this cancer in terms of their severity and symptoms is crucial for improving treatment (Nguyen *et al*., 2009; Trevisani *et al*., 2007). Essentially, there are two subtypes of this cancer, which does not provide a precise and distinct classification. Therefore, many studies have sought to incorporate other types of information to improve subtype identification. Yamashita *et al*. (2008) used the EpCAM co-expression signature, as well as gene expression, to classify hepatocellular carcinoma (HCC),

the most common type of primary liver cancer. As a result, the study identified two distinct subtypes of HCC, EpCAM+ and EpCAM-, and also studied a specific set of genes. The gene expression patterns of these genes showed a high ability to predict the survival time of HCC patients, the study also relied on the integration of several types of genomic data, including DNA gene expression (mRNA), copy number variations (CNVs), miRNA gene expression, and CpG loci, as well as clinical data, in order to identify subtypes of hepatocellular carcinoma, which is the most common type of cancer. (Chaudhary *et al*., 2018). Recently, researchers have become increasingly interested in studying the role of genetic variants in complex diseases, due to their importance in understanding the molecular mechanisms underlying the occurrence and development of diseases, including neurocognitive disorders. (Alinejad-Rokny *et al*. 2020).

Galangal (*Alpinia officinarum*) is an important aromatic plant belonging to the ginger family (Zingiberaceae). Galangal is cultivated in Southeast Asian countries such as China and Indonesia. It is used in food manufacturing as a natural flavoring and preservative due to its chemical composition, which is effective against many microorganisms that cause food spoilage and decay. Galangal is also an antioxidant and an anti-cancer agent, and it is effective against many diseases (Zhou *et al*, 2018). Galangal rhizome is widely used as a cooking spice and medicinal ingredient, galangal is also a distinctive taste and aroma ingredient, adding galangal to food or drinks not only enhances the flavor but also boosts the body's immune system due to its ability to fight free radicals (Sartika *et al*., 2026). Galangal contains bioactive components that are safe for continuous consumption in certain amounts. It has antimicrobial properties, in addition to its therapeutic benefits and use as a traditional medicine for treating skin diseases, digestive disorders, diarrhea, and as a functional skin moisturizer (Hamad *et al*., 2023; Budiman *et al*., 2013).

This study aimed to evaluate galangal rhizome and its potential use as an adjunctive or natural alternative treatment for cancer, given its high efficacy and excellent selectivity, as well as its role in combating cancer cells through programmed cell death naturally without affecting normal cells.

Materials and Methods

Sample Collection, Cleaning, and Impurity Removal: Samples were collected from local markets in the city of Kufa, Najaf Governorate, Iraq. Impurities and dirt were then manually removed from the samples, which were washed with water and dried using an electric drying oven at 40°C for 24 hours.

Preparation of galangal powder: The galangal rhizome powder was prepared by grinding the sample well and drying the sample in a drying oven at 40°C for 24 hours. Then it was packed in polyethylene bags and kept at a temperature of (-18°C) until use.

Estimation of the chemical composition of galangal: The chemical composition of the samples used under study, namely moisture, fat, protein, carbohydrates, and ash, was estimated based on the method described by (A.O.A.O, 2016).

Aqueous-alcoholic extraction of galangal rhizome: The cold aqueous extract of the galangal rhizome powder under study was prepared to obtain the bioactive substance according to the method described by Ojeda *et al*. (2022), while the alcoholic extract was prepared according to the method described by Tungal *et al*. (2025).

Estimating the yield percentage: The yield percentage was estimated according to the method described by Alcazar *et al*., (2017).

Estimation of total phenolic content: The concentration of total phenolic compounds of the aqueous and alcoholic extract of galangal rhizome powder was measured using Folin ciocalteu reagent according to the method described by Laouini and Ouahrani (2017) and based on the standard curve for gallic acid, while preserving the heat-sensitive active compounds.

Estimation of total flavonoid content: The total flavonoids in both aqueous and alcoholic extracts of galangal rhizome powder were estimated following the method described by Sen *et al*. (2013), and based on the standard curve for quercetin.

Measurement of antioxidant activity: The antioxidant activity of both the aqueous and alcoholic extracts of the samples under study was estimated based on both free radical inhibition activity and reducing power.

Free radical inhibition efficacy: The free radical inhibition ability of aqueous and alcoholic galangal rhizome powder extracts was measured using the (DPPH) 2,2-Diphenyl-1-picrylhydrazyl assay following the method mentioned by Laohakunjit *et al*., (2017) with some modification, and the synthetic antioxidant (BHT) prepared at a concentration of (0.1 mg/ml of ethanol) was adopted as a sample for comparison.

Reducing power determination: The reducing power of the aqueous and alcoholic extracts of galangal rhizome was assessed according to Li *et al*. (2015).

Culture and development of Cancer Cell Lines (HepG2, Caco-2, MCF-7)

Cancer cell lines (HepG2, Caco-2, MCF-7) and normal human cells (WRL68) were cultured according to the method described by Chandra *et al*. (2022).

The percentage of cell viability in the sample was calculated using the following equation:

Percentage of vitality of living cells = [(Living cells)/(Dead cells)] × 100

MTT Cytotoxicity Test for Cell Viability: This test was performed to select the optimal cancer cell line for High Content Screening (HCS). Cytotoxicity was tested using the MTT reagent to determine the cytotoxic effect of the aqueous and alcoholic extracts against HepG2, Caco-2, MCF-7, and WRL68 cancer cells for comparison. Different concentrations of the extracts (12.5, 25, 50, 100, 200, and 400 µg/mL) were used, and absorbance was measured at 570 nm. IC50 values for the samples were calculated using the following equation:

Viability (%) = (Optical density of sample) / (Optical density of control) × 100

High Content Screening (HCS) Test: The cytotoxicity of galangal rhizome extracts against HepG2 hepatoma cells was evaluated using the method described by Al-Shaikh and Doosh (2024), with some modifications to study the effect of galangal extracts based on the results obtained from the MTT test. Measurement and monitoring of changes included cellular indices such as cell count, cell membrane permeability, mitochondrial membrane permeability, and cytochrome c release. All treatments were compared with untreated control cells.

Results and discussion

Chemical Composition of Galangal

Table (1) shows the results of the chemical composition of the galangal rhizome, where the percentages of moisture, protein, fat, ash, carbohydrates, and fiber under study reached approximately (4.77, 4.55, 5.39, 5.21, 80.08, 19.60) % respectively. Some of these results were in agreement with some researchers and differed with others. They were in agreement with what Aljobair, (2022) concluded and were also close to what Eid *et al.* (2022) and Tarannum *et al*., (2025) found respectively. This slight difference between the studies is attributed to the differences in the type of galangal used and its state (fresh or dried powder), the geographical location of its cultivation, the conditions of its processing after harvest, and the conditions of sample preparation and analysis (Aljobair 2022).

Table (1) Chemical composition of galangal rhizome

Components of galangal rhizome	Percentage
Moisture	% 4.77
Protein	% 4.55
Fat	% 5.39
Ash	%5.21
Carbohydrate	% 80.08
Fiber	%19.60
pH	4.51

Each number in the table represents the average of three replicates

Estimation of the Percentage Yield of Galangal Rhizome

Table (2) shows the percentage yield of the aqueous and alcoholic extracts of galangal rhizome. The aqueous extract recorded a yield of 18%, while the alcoholic extract yielded 24% per 200g of dry galangal. These results are similar to those found by Bouhenouche (2025), who found yields of 13.8% and 14.8% for the aqueous and alcoholic extracts, respectively. The difference in the percentage yield is attributed to the difference in the polarity of the solvent, the solubility of the compounds in the aqueous and alcoholic solvents used in the extraction, the presence of bioactive components in the sample, pressure, extraction time, and temperature (Herrera-Pool *et al*., 2025).

Table (2) Percentage yield of aqueous extract and alcoholic extract

Type of Extract	% Yield	pH
Aqueous extract of galangal rhizome	18	5.41
Alcoholic extract of galangal rhizome	24	5.43

Estimation of the total phenolic and flavonoid content in the aqueous and alcoholic extracts of galangal rhizome powder

Figure (1) shows the quantity of phenolic compounds and flavonoids in the aqueous and alcoholic extracts of the galangal rhizome powder under study. Statistical analysis revealed significant differences in the quantity of phenolics and flavonoids between the extracts. The highest quantity of phenolics and flavonoids was found in the alcoholic extract, reaching 63.19 mg/g of phenolics and 36.22 mg/g of flavonoids. In contrast, the aqueous extract recorded 59.96 mg/g of phenolics and 31.61 mg/g of flavonoids. This result was consistent with what Wasel *et al*. (2025) reported, as they found that the content of phenolic compounds in the alcoholic extract of galangal rhizome was 0.39 ± 59.71 mg gallic acid/g dry weight, while the amount of flavonoids was 0.13 ± 25.99 mg gallic acid/g. The difference in the amount of phenolic compounds between the aqueous and alcoholic extracts is attributed to the nature of the separated compounds and their solubility in the solvents used in the extraction (Ivanović, *et al*., 2021).

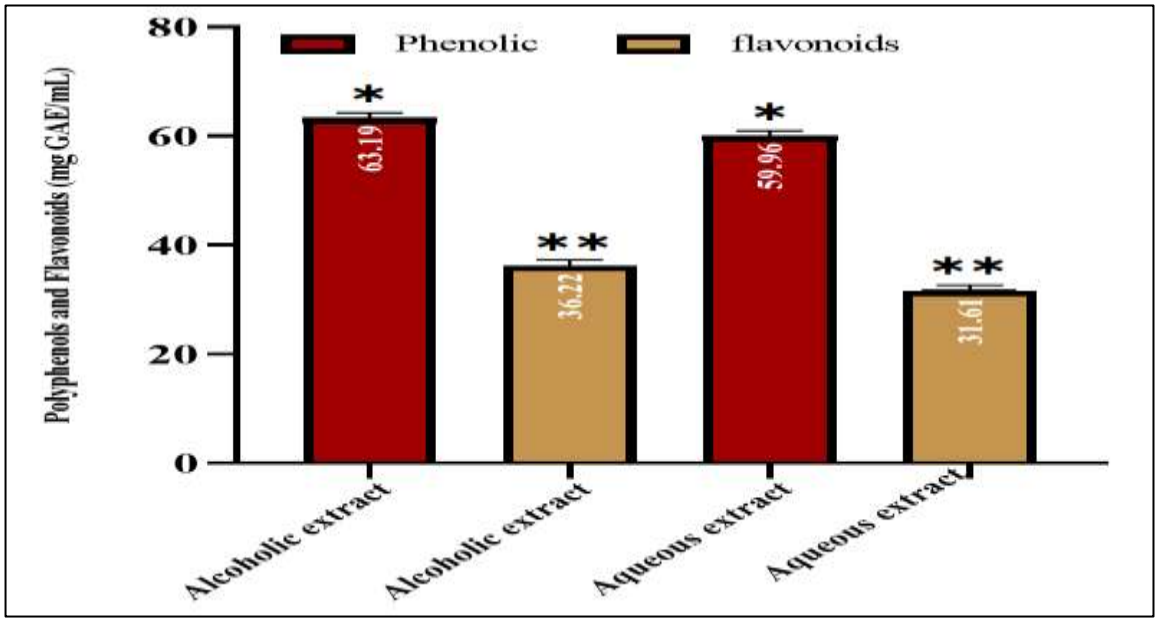


Figure (1) Concentration of phenolic and flavonoid compounds in the aqueous and alcoholic extract of galangal rhizome powder

Antioxidant activity of aqueous and alcoholic extracts of galangal rhizome powder

1-Free radical scavenging activity using DPPH reagent

Table (3) shows the percentage of free radical inhibition activity of aqueous and alcoholic extracts of galangal rhizome powder using the reagent (DPPH), which turns from dark purple to yellow when reduced, and the absorption is measured at 517 nm. Four concentrations were used and compared with the synthetic antioxidant (BHT) Butylated hydroxytoluene. The results showed that the percentage of free radical scavenging activity of the aqueous extract was (70.20, 75.77, 83.32, 84.97)%, while the percentage of free radical scavenging activity of the alcoholic extract was (77.53, 78.10, 84.85, 85.49)% at concentrations of (0.12, 0.25, 0.50, and 1) mg/ml respectively. The results of the statistical analysis show that the alcoholic extract significantly outperformed the aqueous extract in free radical scavenging activity at some concentrations. This superiority may be attributed to the high percentage of some active compounds in the alcoholic extract, including phenols, flavonoids, terpenes, tannins, saponins, and alkaloids, as well as other factors such as the type and nature of the solvent, extraction methods, and the concentration and nature of phenolic compounds in plants (Mardhiyyah *et al*., 2021). These results are consistent with several previous studies (Quoc and Pham, 2021; Aljobair, 2022; Fitasari *et al*., 2025) that found that galangal rhizome has high activity in the ability to inhibit free radicals, with percentages of (80.06, 77.76, 79.98)% respectively.

Table (3) Ability of aqueous and alcoholic extracts of galangal rhizome to inhibit free radicals

Sample concentration	Aqueous extract %	Alcoholic extract %	%BHT	(P≤0.05) LSD
0.12 mg/ml	70.20A	77.53B	C 81.15	1.934*
0.25 mg/ml	75.77A	78.10B	C 85.05	1.984*
0.5 mg/ml	83.32A	84.85A	B 89.28	1.636*
1 mg/ml	84.97A	85.49A	B 92.77	1.473*
LSD (P≤0.05)	1.679*	1.791*	1.521*	----

* Significant differences exist

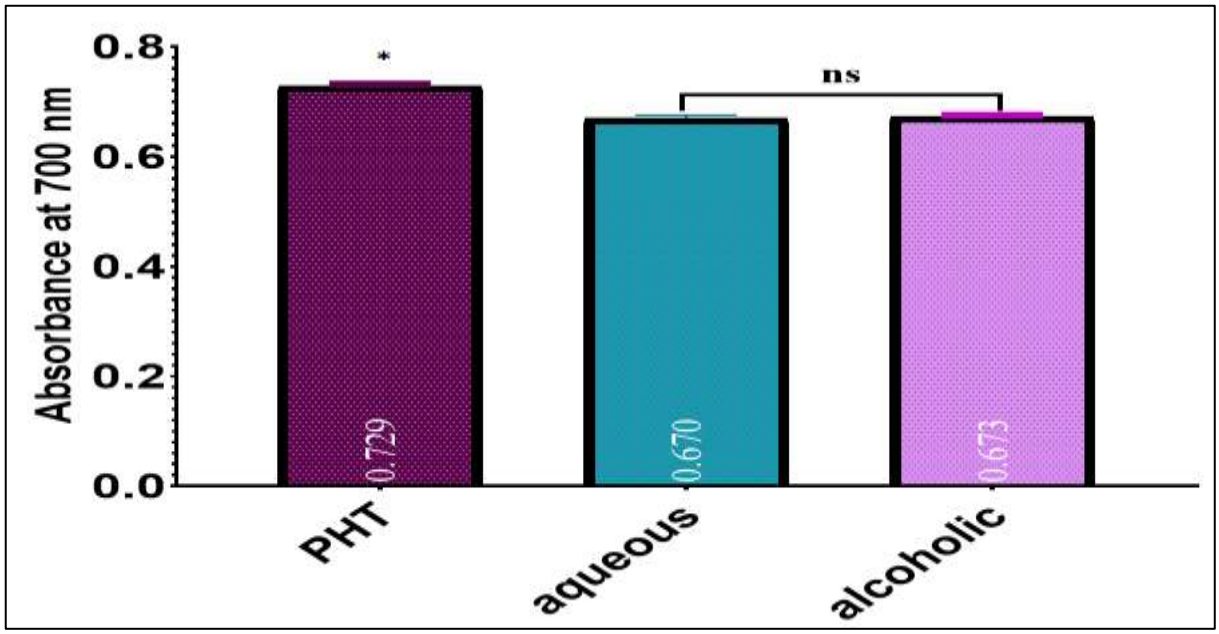
Based on the results obtained in this study, it is evident that galangal's secondary metabolites, such as phenols, terpenes, alkaloids, and others, possess antioxidant and antimicrobial properties, making them suitable for use in food and pharmaceutical products, such as galangal oil-based nanoemulsions, active food packaging, clinical anesthetics, and aromatherapy slimming treatments (Zhou *et al*., 2021; Destryana *et al*., 2025).

2- Reducing Power

Figure (2) shows the reducing power of both the aqueous and alcoholic extracts of galangal rhizome powder compared to the synthetic antioxidant (BHT), (estimated as an absorbance at a wavelength of 700 nm). The results of the statistical analysis indicate that there are no significant differences at the probability level (P≤0.05) between the aqueous and alcoholic extracts, as the reducing power of the alcoholic extract reached 0.670 nm, while the aqueous extract obtained a reducing power of 0.673 nm. When compared to BHT, we note its significant superiority at the probability level (P≤0.05), as it recorded a higher reducing power than the aqueous and alcoholic extracts, amounting to 0.729 nm. This is expected given that they are synthetic antioxidants. Given that galangal rhizome extract is a natural substance considered safer than BHT, health considerations must be taken into account when using synthetic antioxidants in processed food products. Numerous studies have pointed to their many negative side effects that harm consumers (Hefnawy *et al*., 2016). This finding aligns with that of Wasel *et al*. (2025), who indicated that the alcoholic extract exhibited the highest reducing power of 0.689 nm, and is

consistent with the findings of Juntachote and Berghofer (2005), who reported a reducing power of 0.75 nm for the alcoholic galangal extract.

Figure (2) Reducing power of aqueous and alcoholic extracts of galangal rhizome powder compared to BHT



Based on the results of the ability to inhibit free radicals using DPPH reagent and the reducing power of the aqueous and alcoholic extracts of the galangal rhizome under study, it was shown that the efficiency of the extracts led to the production of effective compounds with high ability to inhibit free radicals and reducing power.

Biological activity Cytotoxicity test of aqueous and alcoholic extracts of galangal rhizome against three types of cancer cells.

This test was conducted to determine the ability of the aqueous and alcoholic extracts of galangal rhizome to inhibit the growth of cancer cells in vitro, using the MTT cytotoxicity assay to determine the toxic effect of the extracts on three types of cancer cells: HepG2 liver cancer cells, MCF-7 breast cancer cells, and CaCo-2 colon cancer cells, and to compare them with normal WRL68 cells. The cancer cells were treated with different concentrations of the aqueous and alcoholic extracts of galangal rhizome (12.5, 25, 50, 100, 200, and 400 µg/ml) at three replicates per concentration for 24 hours at 37°C.

Cytotoxic effect of aqueous and alcoholic extracts of galangal rhizome against HepG2 cells, CaCo-2 cells, and MCF-7 cells

The cytotoxicity results shown in Table (4) indicate that the aqueous and alcoholic extracts of galangal rhizome exhibited an inhibitory effect against HepG2 liver cancer cells, with inhibition rates of (10.27, 24.50, 37.00, 47.88, 60.77, 68.29)% and (6.83, 12.78, 24.50, 35.34, 49.04, 58.99)% for the aqueous and alcoholic extracts respectively for the concentrations used (12.5, 25, 50, 100, 200, 400 µg/mL) respectively. The aqueous and alcoholic extracts of galangal rhizome also exhibited an inhibitory effect against CaCo-2 colon cancer cells, with inhibition rates of (5.52, 11.96, 24.43, 36.19). (48.23, 58.72) % and (5.91, 11.77, 23.69, 34.57, 47.84, 58.72) % for the aqueous and alcoholic extracts respectively, and for the same concentration mentioned. As for the aqueous and alcoholic extract of galangal rhizome, it showed an inhibitory effect against MCF-7 breast cancer cells, with an inhibition rate of (3.28, 4.33, 5.48, 13.47, 22.73, 35.61) % and (1.74, 3.32, 4.48, 6.06, 12.81, 25.31) % for the aqueous and alcoholic extracts respectively, for the concentrations used (12.5, 25, 50, 100, 200, 400 µg/mL) respectively, while the inhibition percentage of normal WRL68 cells for the same substance was (1.93, 2.59, 3.79, 4.98, 12.89, 26.55)% and (1.66, 2.55, 4.06, 5.41, 6.72, 15.13)% for both extracts at the same concentrations respectively. This result is consistent with what Aziz *et al*. (2024) found, which showed that extracts of galangal rhizome had a significant toxic effect against HepG2 cancer cells and MCF-7 breast cancer cells. It is also consistent with what Kumar *et al*. (2007) found, which showed that the cell viability of colon cancer cells reached 52% at a concentration of 50 µg/mL.

Table (4) Survival rate of HepG2, CaCo-2, and MCF-7 (ex vivo) treated with different concentrations of aqueous and alcoholic extracts of galangal compared to normal WRL68 cells using the MTT test for 24 hours at a temperature of 37°C.

Type of Extract	Call Line	Viability% SD± mean (µg/mL)					
		12.5	25	50	100	200	400
Aqueous	HepG2	89.73±1.53	75.50±1.77	63.00±1.46	52.12±0.69	39.23±2.58	31.71±1.33
	CaCo-2	94.48±0.43	88.04±2.80	75.57±1.91	63.81±1.80	51.77±1.62	41.28±1.33

	MCF-7	96.72±0.24	95.67±0.52	94.52±0.26	86.53±1.35	77.27±1.68	64.39±2.58
	WRL68	98.07±0.58	97.41±0.29	96.21±0.40	95.02±0.94	87.11±2.03	73.45±0.75
Alcoholic	**HepG2**	93.17±0.46	87.22±3.15	75.50±2.45	64.66±1.99	52.16±3.09	41.28±2.57
	CaCo-2	94.09±0.30	88.23±1.11	76.31±1.57	65.43±0.96	50.96±1.73	41.01±1.33
	MCF-7	98.26±0.23	96.68±067	95.52±046	93.94±0.35	87.19±2.40	74.69±2.75
	WRL68	98.34±0.24	97.45±0.30	95.94±0.81	94.59±0.24	93.28±0.30	84.87±1.34

As shown in Figure (3), the IC50 values of the half-maximal inhibitory constant (IC50) for HepG2 liver cancer cells and normal WRL68 cells when treated with the extracts showed a highly significant difference at a probability level of (0.05 p<), as the IC50 values of the aqueous extract reached 110.52 µg/mL for liver cancer cells compared to 327.2 µg/ml for normal cells, while the alcoholic extract reached 111.1 µg/mL and 201.4 µg/ml for normal cells and liver cancer cells, respectively. The results showed a highly significant difference at a $p < 0.05$, with the IC50 values for the aqueous extract reaching 91.45 µg/mL for colon cancer cells compared to 327.2 µg/mL for normal cells. The alcoholic extract, on the other hand, had IC50 values of 104.2 µg/mL and 201.4 µg/mL for normal cells and colon cancer cells, respectively. Similarly, at the same $p < 0.05$, the IC50 values for the aqueous extract reached 247.9 µg/mL for breast cancer cells compared to 327.2 µg/mL for normal cells, while the alcoholic extract had IC50 values of 165.95 µg/mL and 201.4 µg/mL for normal cells and breast cancer cells, respectively. This result is similar to what Da'i et al. (2019) found, as he found that galangal extract showed an inhibitory effect against colon cancer cells with an IC50 value of 66.11 µg/mL. He indicated that the alcoholic extract showed strong activity against colon cancer because it contains the compound 1'-acetoxychavicol acetate, which is a compound with very high toxic activity against the cancer cells that were studied, namely breast cancer type T47D, cervical cancer cells HeLa, and colon cancer cells WiDr.

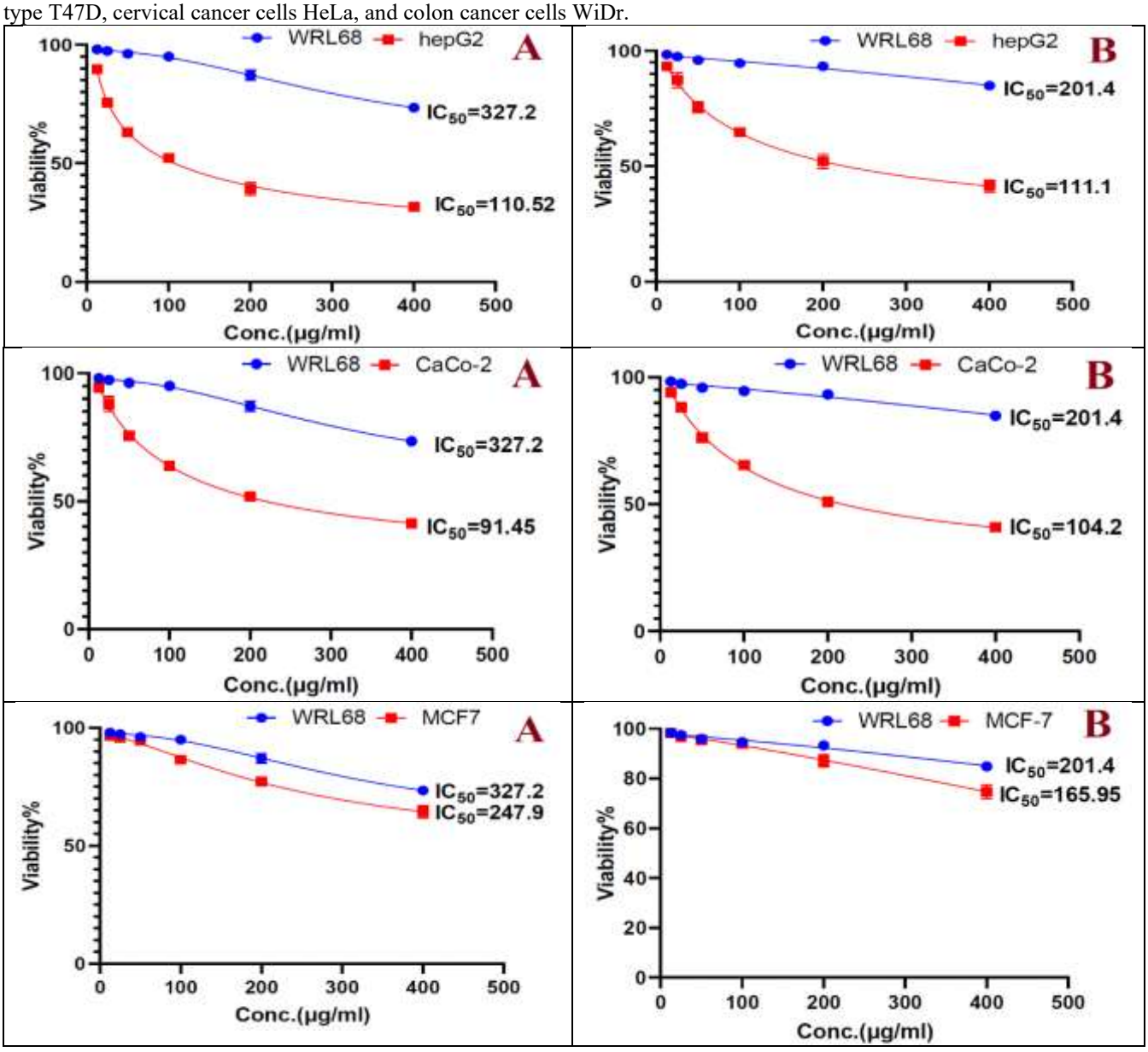


Figure (3) The toxic effect of the aqueous (A) and alcoholic (B) extracts of galangal on the cancer cell lines HepG2, CaCo-2, and MCF-7 compared to the normal cell line WRL68 using the MTT assay

Cytotoxicity Assay Study of the toxic effect of aqueous galangal extract on the cancer cell line HepG2 using High Content Screening (HCS) assay

This test was performed using the HCS device after performing the MTT test on the aqueous and alcoholic extract of galangal rhizome on three cancer lines, namely the liver cancer line HepG2, the colon cancer line CaCo-2, and the breast cancer line MCF-7, and comparing them to normal WRL68 cells at different concentrations. Based on the results of the MTT test, the aqueous extract and the cells of the liver cancer line HepG2 were chosen to complete the cytotoxicity tests as it gave the highest inhibition rate.
The cytotoxicity of the selected aqueous extract at certain concentrations against HepG2 cancer cells was studied by performing some tests using the (HCS) device to detect the morphological changes that may occur to the cells. The cellular indicators represented (cell number, total nuclear density estimate, cell membrane permeability, mitochondrial membrane permeability, cytochrome c release level) were measured and compared with untreated cells.

Cell viability (VCC)
The results of the cell viability assessment shown in Table (5) and Figures (4) and (9) indicate that the aqueous extract of galangal rhizome had a highly significant effect on the number of HepG2 cells. Statistical analysis revealed a highly significant decrease in the number of viable cells, specifically at concentrations of 100 and 200 µg/ml, reaching 34.48% and 38.22%, respectively, compared to the control treatment. This finding is consistent with that of Kim *et al*. (2023), who reported that the aqueous extract of galangal rhizome reduced the viability of liver cancer cells in a dose-dependent manner, with an IC50 value of 67 after 24 hours. This result was lower than that of Melanathuru *et al*. (2017), who indicated that the aqueous extract of galangal rhizome led to the death of 88.36% of HepG2 cells.

Table (5) Cellular activity of aqueous galangal extract at different concentrations on the viability of HepG2 cancer cell lines

µg\ml Treatment	Cell viability (VCC)		Significant	P. value
	SD	Mean		
Control	119.91	3348.3	—	—
25	92.22	3335.6	Ns	0.9992
50	86.64	3227.0	Ns	0.3881
100	70.40	2193.6	****	<0.0001
200	102.65	2068.3	****	<0.0001
				قيمة L.S.D (p< 0.05)

Ns - No significant difference, ** Highly significant difference, ** Very high significant difference.**

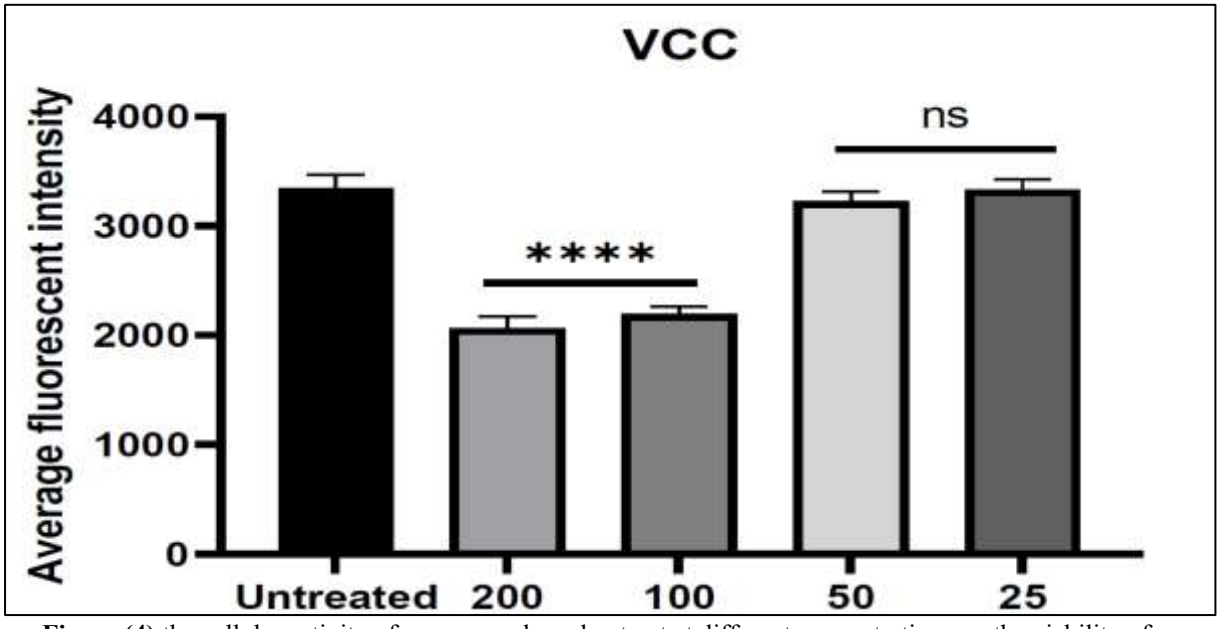


Figure (4) the cellular activity of aqueous galangal extract at different concentrations on the viability of HepG2 cancer cell lines after 24 hours of incubation at a temperature of 37°C using an HCS device.

Total Nuclear Intensity (TNI)
Estimating total nuclear density is important because nuclear morphology is a key characteristic of apoptosis in cancer cells. Hoechst dye binds to DNA fragments, thus determining the effect of apoptosis (Kuppusamy et al., 2023). The results shown in Table 6 and Figures 5 and 9 indicate some morphological changes in HepG2 cancer cells, showing an increase in nuclear volume and cell membrane permeability. The results also showed that the aqueous extract of galangal rhizome had a clear effect against HepG2 cells, which in turn led to a significant increase in nuclear density. The highest nuclear density was observed at concentrations of 100 and 200 µg/ml, with increases of 26.85% and 66.88%, respectively, compared to the control treatment. Aziz et al., (2024) reported

that programmed cell death is a morphological process that involves DNA destruction and cell shrinkage, and is controlled by a series of molecular processes that can occur internally or externally.

Table (6) Cellular activity of aqueous galangal extract at different concentrations on the nuclear density of HepG2 cancer cell lines

µg\m Treatment	Nuclear Intensity (TNI)		Significant	P. value
	SD	Mean		
Control	17.05	449.0	—	—
25	12.01	425.3	Ns	0.1225
50	8.32	458.6	Ns	0.7476
100	1.15	569.6	****	<0.0001
200	16.25	749.3	****	<0.0001
				قيمة L.S.D (p< 0.05)

Ns - No significant difference, ** Highly significant difference, ** Very high significant difference.**

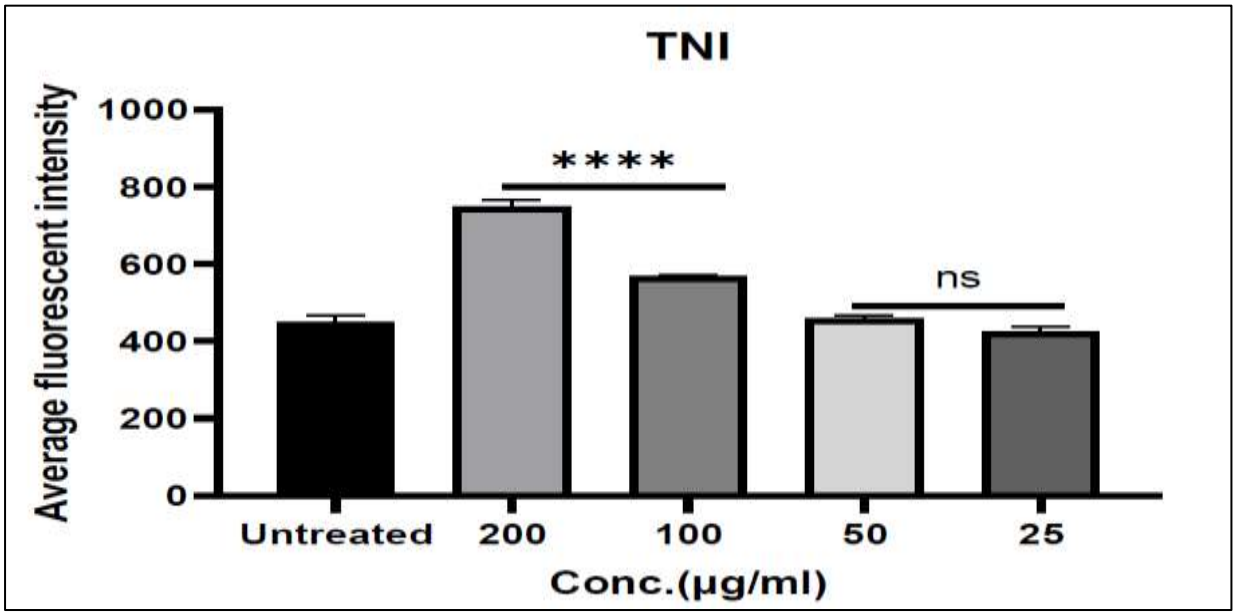


Figure (5) The cellular activity of aqueous galangal extract at different concentrations on the nuclear density of HepG2 cancer cell lines after 24 hours of incubation at a temperature of 37°C using an HCS device.

Cell Membrane Permeability (CMP)

Cell membrane permeability is an important indicator of cell integrity and plays a crucial role in physiological and pathological processes as the first line of defense for cells. The membrane's function is not limited to the transfer of information and materials between cells; it can also sense the stimulation of external toxic substances, triggering an immediate response such as changes in morphology, structure, or components (Niu et al., 2023; Al-Shaikh and Doosh, 2024). The results shown in Table (7) and Figures (6) and (9) demonstrate the effect of the aqueous extract of galangal rhizome at different concentrations against HepG2 liver cancer cells via a multi-stage mechanism. This mechanism begins by disrupting the oxidative balance within the cell, which correlates with the antioxidant activity in the DPPH assay. Consequently, its effect extends to the mitochondrial membrane, causing increased release of cytochrome C into the cytoplasm, which leads to activation of the internal pathway of programmed cell death. The results indicate a significant increase in membrane permeability at a concentration of 200 µg/ml. This was reflected in the integrity of the plasma membrane, and these changes are consistent with the observed decrease in cell viability, represented by the previously mentioned IC50 value. This suggests that the aqueous extract of galangal rhizome not only inhibits the growth of cancer cells but also stimulates mitochondrial-dependent apoptosis mechanisms. Therefore, galangal is considered a promising source of bioactive compounds with anticancer activity. These results are consistent with what Fiorucci *et al*. (2023) stated, as they reported that the anticancer effect of galangal extract is not limited to inhibiting cell growth but extends to stimulating mitochondrial-dependent programmed cell death. The results also indicated no significant differences for the concentrations of 25, 50, and 100 µg/ml respectively compared to the control treatment. A significant difference was observed at the (P< 0.05) level in cell membrane permeability at 200 µg/ml, as the percentage in cell membrane permeability reached 99.10% compared to the control treatment.

Table (7) Cellular activity of aqueous galangal extract at different concentrations on cell membrane permeability of HepG2 cancer cell lines

µg\ml Treatment	Cell Membrane Permeability (CMP)		Significant	P. value
	SD	Mean		

Control	9.01	133.6	—	—
25	5.13	137.6	Ns	0.8508
50	5.50	150.6	Ns	0.0257
100	2.08	140.3	Ns	0.5332
200	7.54	266.0	****	<0.0001
(p< 0.05) L.S.D قيمة				

Ns - No significant difference, ** Highly significant difference, ** Very high significant difference.**

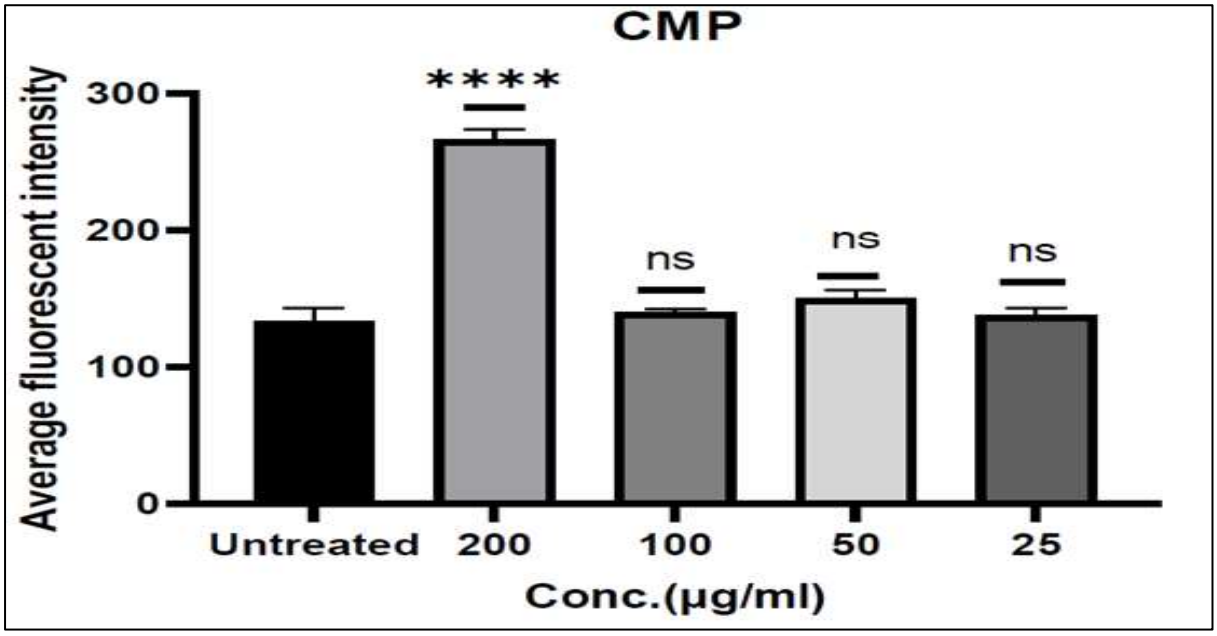


Figure (6) the cellular activity of aqueous galangal extract at different concentrations on the nuclear density of HepG2 cancer cell lines after 24 hours of incubation at a temperature of 37°C using an HCS device.

Mitochondrial Membrane Potential (MMP)

Mitochondrial membrane strength is an important indicator of mitochondrial integrity, and depolarization and changes in the mitochondrial membrane are ideal indicators of dysfunction related to mitochondrial membrane permeability. This leads to increased mitochondrial membrane permeability and the release of cytochrome c, which then stimulates the mechanism of drug-induced programmed cell death (Aghaei-Zarch *et al*., 2023).

The results shown in Table (8) and Figures (7) and (9) indicate that the aqueous extract at different concentrations showed a highly significant effect at the level of (P<0.05), which led to phenotypic changes in the shape and size of the cell membranes of HepG2 liver cancer cells depending on the concentration. It then affected the mitochondrial membrane, especially at concentrations of 100 and 200 µg/ml, by a percentage of 26.45% and 31.03% respectively, compared to the control treatment. The results show a decrease in the strength of the mitochondrial membrane, thus allowing the transfer of molecules and ions, interrupting the respiratory chain, releasing cytochrome C, and then programmed cell death of HepG2 cancer cells due to the effect of the extract obtained from the galangal rhizome. The results were consistent with those of Kim *et al*. (2023), who found that galangal extract led to concentration-dependent mitochondrial membrane stress. They observed a significant increase in liver cancer cell death (26.93%) compared to normal and treated cells (3.88%), indicating that programmed cell death damaged the mitochondrial membrane and thus increased its permeability. Kato *et al*. (2014) reported that the formation of reactive oxygen species (ROS) is a byproduct of oxygen consumption in mitochondria, generated by specialized plasma membrane oxidants (NADPH), during programmed cell death in cancer cells.

Table (8) Cellular activity of aqueous galangal extract at different concentrations on the strength of the mitochondrial membrane of HepG2 cancer cell line

µg\ml Treatment	Mitochondrial Membrane (MMP)		Significant	P. value
	SD	Mean		
Control	10.53	562.0	—	—
25	9.00	542.0	Ns	0.6484
50	46.11	484.3	**	0.0051
100	6.80	413.3	****	<0.0001
200	8.32	387.6	****	<0.0001
(p< 0.05) L.S.D قيمة				

Ns - No significant difference, ** Highly significant difference, ** Very high significant difference.**

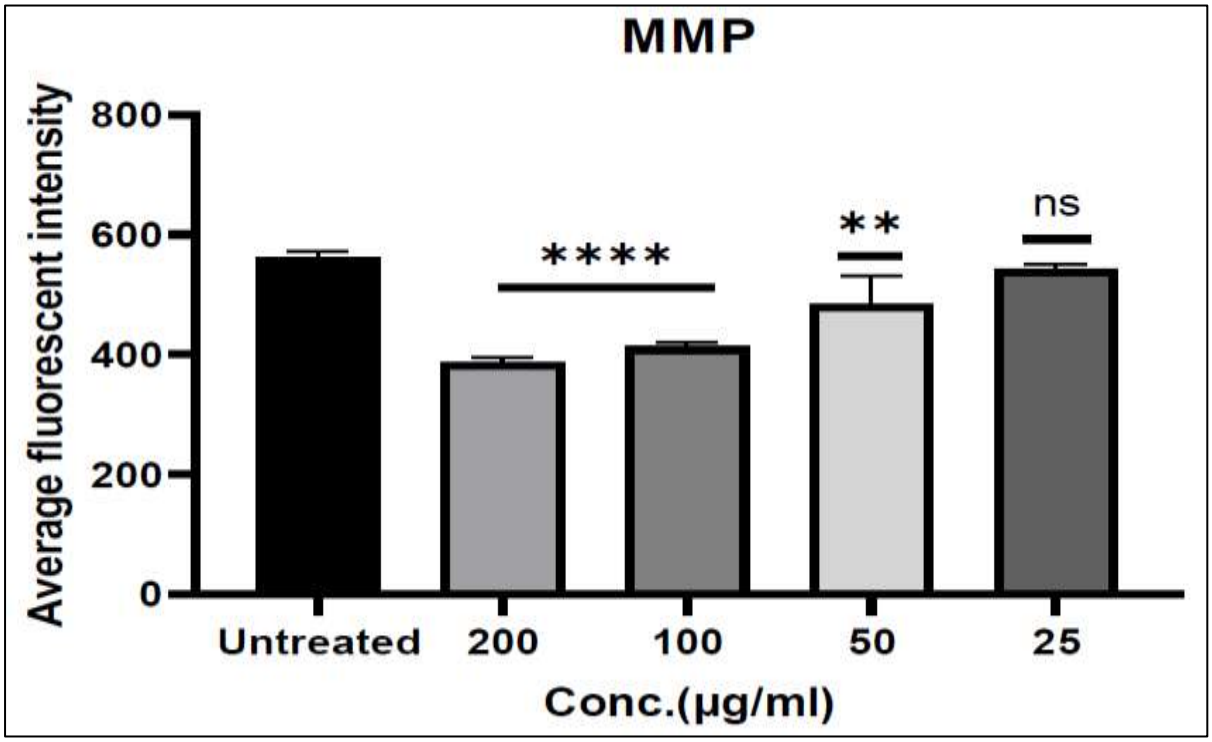


Figure (7) The cellular activity of aqueous galangal extract at different concentrations on the strength of the mitochondrial membrane of HepG2 cancer cell line after 24 hours of incubation at a temperature of 37°C using an HCS device.

Cytochrome C Releasing (CC)

Cytochrome c is a relatively small globular protein that is a major component of the electron transport chain and is loosely bound to the outer layer of the inner mitochondrial membrane. When released from the membrane, it plays a key role in programmed cell death (Brazhe *et al*., 2023).

The results shown in Table (9) and Figures (8) and (9) a significant increase in the level of mitochondrial cytochrome-C released in HepG2 liver cancer cells treated with the aqueous extract of galangal rhizome when using the concentrations of 100 and 200 µg/ml compared to the control treatment, as the percentage reached 47.67% and 62.63% respectively. This increase is highly significant at a probability level ($P < 0.05$) compared to the control treatment and the two treatments with concentrations of 25 and 50 µg/ml, while the concentrations of 25 and 50 µg/ml did not record significant differences. The results indicate that the aqueous extract stimulated the mitochondrial-dependent intrinsic pathway of apoptosis in a concentration-dependent manner. Disruption of mitochondrial membrane permeability leads to the release of cytochrome C and activation of caspase chain responsible for programmed cell death. These findings are consistent with what is known about the active compounds in galangal that possess anticancer effects by inducing apoptosis and inhibiting cancer cell growth.

The release of cytochrome c from the mitochondrial membrane of HepG2 cancer cells due to the effect of galangal extract on mitochondrial membrane strength, through activation of the mitochondrial apoptosis pathway, thus contributing to cell growth inhibition by inducing apoptosis and reducing cell proliferation and survival, is consistent with the findings of Ruttanaphan *et al*. (2020), who found that galangal extract helped reduce mitochondrial membrane strength, thereby releasing cytochrome c into the cytoplasm and releasing it from the mitochondrial membrane through mitochondrial permeability transmembrane pores, thereby initiating a cascade of events leading to apoptosis. This also aligns with the findings of Kim *et al*. (2023), who indicated that galangal extract possesses anticancer activity by inhibiting cell cycle progression, leading to apoptosis in hepatocellular carcinoma cells.

Table (9) Cellular activity of aqueous galangal extract at different concentrations on cytochrome c release in HepG2 cancer cell lines

µg\ml Treatments	Cytochrome C Releasing (CC)		Significant	P. value
	SD	Mean		
Control	**7.37**	**412.6**	—	—
25	**7.02**	**409.6**	Ns	**0.9932**
50	**8.54**	**419.0**	Ns	**0.9102**
100	**19.39**	**609.3**	****	**<0.0001**
200	**12.52**	**671.0**	****	**<0.0001**
(p< 0.05) L.S.D قيمة				

Ns - No significant difference, ** Highly significant difference, ** Very high significant difference.**

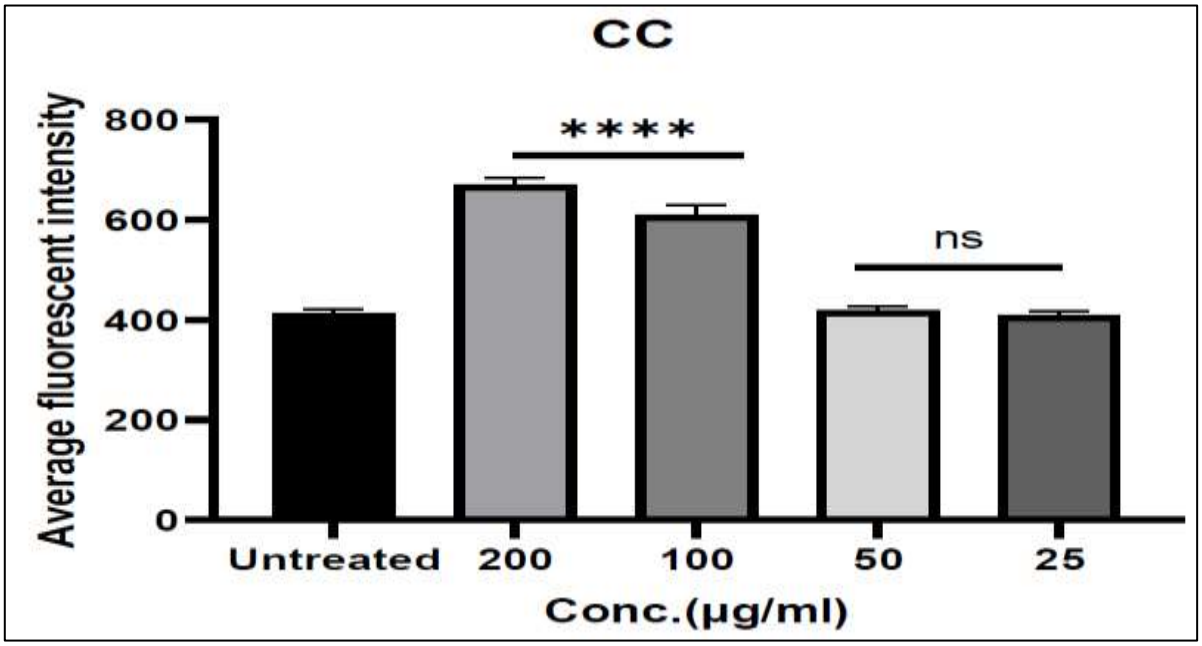


Figure (8) The cellular activity of aqueous galangal extract at different concentrations on the release of cytochrome C from HepG2 cancer cell lines after 24 hours of incubation at a temperature of 37°C using an HCS device.

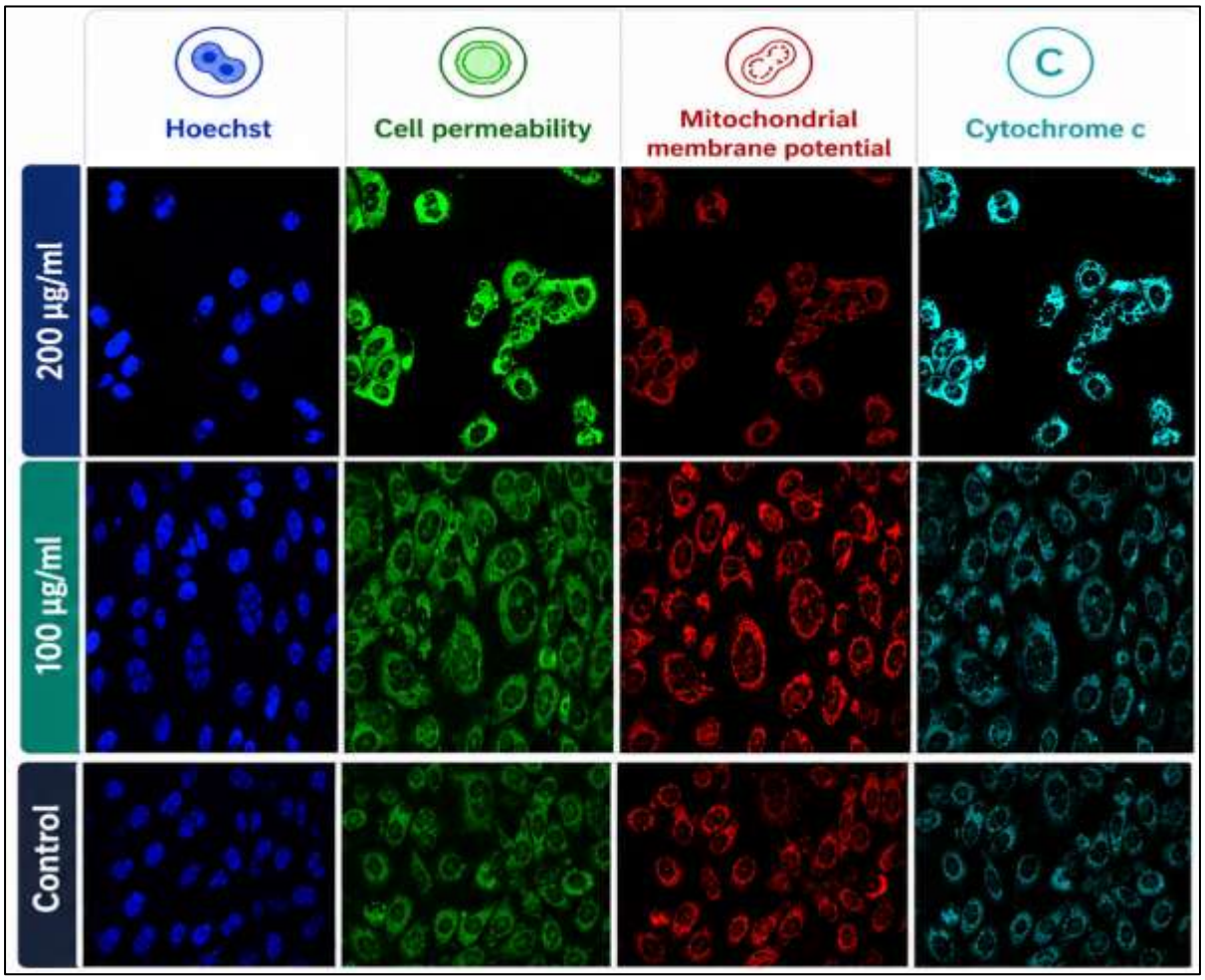


Figure (9) Cytotoxicity analysis using the HCS assay of HepG2 cancer cells treated with different concentrations of aqueous extract of galangal rhizome for 24 hours at 37°C

Conclusions

Based on the results of this study, we can conclude that the aqueous and alcoholic extracts of galangal rhizome contain a high percentage of phenolic compounds and flavonoids, thus possessing biological properties in inhibiting free radicals and exhibiting high reducing power. This study showed that the aqueous and alcoholic extracts of galangal rhizome contain biologically active compounds that inhibit the growth and proliferation of HepG2, CaCo-2, and MCF-7 hepatocellular carcinoma cells through apoptosis (programmed cell death).

Furthermore, the cytotoxicity results using HCS (hepatocellular carcinoma assay) demonstrated that the aqueous extract possesses strong growth-inhibiting properties for hepatocellular carcinoma cells by reducing the number of viable cells, increasing total nuclear density, decreasing mitochondrial membrane permeability, and increasing cytochrome c release. This is achieved by inducing apoptosis through mitochondrial depolarization and DNA fragmentation during the cell cycle.

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