

## Study on The Bioactive Components of *Atriplex halimus L.* and Evaluation of Their Anticancer Effect on Liver Cancer Cells

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### ABSTRACT

**Background:** Liver cancer is one of the most prevalent and fatal malignancies in the human digestive system, killing over 500,000 people each year worldwide.

**Aim of the work:** This study was to analyze for the first time the phytochemical composition of *Atriplex halimus L.* by High-Performance Liquid Chromatography technique and to evaluate its anticancer activity on primary cell culture of human liver carcinoma by MTT assay and compared it with a chemotherapy drug.

**Method:** Cancer cells were treated with a concentration of AHE, Cisplatin, and a combination of them. Also, cell viability and morphological changes, Superoxide Dismutase, and Catalase enzyme activity were measured.

**Results:** The results showed two types of flavonols, quercetin, and kaempferol identified. The MTT assay shows the highest cytotoxicity on cancer cells at concentrations of 100 and 200 µg/ml for all treatments. Superoxide dismutase enzyme showed an increased level (P = 0.001) while catalase enzyme showed decreased level (P = 0.032) in treated cells compared to the untreated cell.

**Conclusion:** Our findings suggest that *A. halimus* possesses antioxidant and anticancer properties, which are possibly due to flavonoids existing in this plant, and could be a potential candidate for the development of drugs for the management of liver cancer cells.

**Keywords:** *Atriplex halimus*, Liver Cancer cell, HPLC, SOD, Catalase.

### INTRODUCTION

The liver is the largest glandular organ that performs several crucial functions to keep the body free from toxins and harmful substances.<sup>(1,2)</sup>

75% of occurrences of liver cancer globally are of the most frequent kind, hepatocellular carcinoma (HCC). The second leading cause of cancer death worldwide and the seventh most prevalent cancer overall is primary liver cancer **Ferlay et al.**<sup>(3)</sup>. Asia and Africa have the highest rates of incidence in the world. China has the most cases due to a high rate (18.3 per 100,000) and the largest population in the world, However, Mongolia has the highest incidence of liver cancer at 93.7 per 100,000 people<sup>(4)</sup>.

Chemotherapy and radiotherapy are among the current treatments. Chemotherapy can put patients under a lot of stress and damage their health. As a result, there is a focus on using alternative cancer treatments and therapies.<sup>(5)</sup>

*Atriplex halimus* is a perennial shrub that is halophytic and thrives in dry and semi-arid climates. It is an excellent species for landscaping in arid and salt-affected areas because of its resilience to severe salinity and drought, where it provides valuable feed for livestock. **Heuzé et al.**<sup>(6)</sup>, **OEP**<sup>(7)</sup>. It can grow from Europe to Northern Africa, western Asia, and W. Iraq and the NE, as well as the Arabian Peninsula **Ghazanfar**<sup>(8)</sup>. Depending on how well it has adapted to the extreme climatic conditions through morphological, anatomical, and physiological changes. The leaves of the plant are used by native herbalists in Arabic countries to cure rheumatism, diabetes, and heart conditions **Said et al.**<sup>(9)</sup>

Some studies highlight the cytotoxic activity of the methanolic extract of *A. halimus*, which had an antiproliferative effect and was highly cytotoxic on cell lines (MCF-7) and (HepG2) **Neima and Abou-Eisha**<sup>(10)</sup>. On the other hand, this extract did not impair the proliferation of normal cell lines. Numerous studies have found plant species utilized in traditional medicine in developing nations that have shown anticancer effects.<sup>(11,12,13,14)</sup>

The present study is a novel study of phytochemical analysis of *A. halimus* by HPLC technique to evaluate its cytotoxic effect on the primary cell culture of human liver carcinoma and assess the anticancer activity of *A. halimus* extract.

### MATERIALS AND METHODS

#### Plant Collection and Extraction

Fresh plants of *Atriplex halimus L.* were collected as a whole plant in April 2022 from the west of Al-Samawa City, Iraq. The plant was identified in the Biology Department, Thi-Qar University, Iraq. The Powder of the whole plant (50gm) was macerated with 250 ml of 80 % methanol using an ultrasonic extractor (20 kHz, power 100w) for 30 min, followed by putting it in a shaker incubator for 24 hours at room temperature.

#### Flavonoids Isolation from *A. halimus*

The methanol soluble fraction (Fr I) was then extracted with second fractionation (Fr II) by adding an equal volume of hexane (100 ml) to the methanol extract and shaking it in a Buchner funnel for four hours. However, the lower layer of the solvent was isolated, and then an equal volume (100 ml) of ethyl

acetate as third fractionation (Fr III) was added to it as in the previous step. The hexane layer of (Fr II) was discarded because it contained the fatty substance, whereas fractions I and III were separated and evaporated by using an evaporator at 40°C<sup>(15)</sup>.

#### **Determination of Total Flavonoid Content (TFC)**

The standard solutions were created by combining 0.5 ml of samples (2 mg/ml in ethanol), 1.5 ml of 95% ethanol (v/v), 0.1 ml of 10% aluminum nitrate Al (NO<sub>3</sub>)<sub>3</sub>, 0.1 ml of 1 mol/l potassium acetate, and 2.8 ml of deionized water. 10% Al(NO<sub>3</sub>)<sub>3</sub> was replaced in the blank with the same amount of deionized water. The calibration curve was created using quercetin at standard concentrations of 25, 50, 80, 100, 150, 200, 250, and 300 mg/100 ml in 80% ethanol (v/v). Spectrophotometric measurements were made of the reaction mixture's absorbance at 415 nm following a 30-minute incubation at room temperature. The analyses were run in triplicates and flavonoids were expressed as Quercetin<sup>(14)</sup>.

#### **High-Performance Liquid Chromatography**

*A. halimus* extract at a concentration of 2 mg/mL in methanol, then (20µL) of methanol extract of *A. halimus* was injected into column C18 (25 cm X 4.6mm) particles of size 5 µm for separation and compared to various standards of flavonoid that were carried out by HPLC **Ding et al.**<sup>(16)</sup>. The separation parameters were determined by the gradient of mobile A (1% CH<sub>3</sub>COOH in HPLC grade water) and mobile B (acetonitrile). However, peak wavelengths were monitored at 272, 280, and 310 nm<sup>(17)</sup>.

#### **Primary cell culture of Human Liver Carcinoma Specimen collection**

Patient sample: a true-cut biopsy was obtained from a female, 28 years old, with liver cell carcinoma. The diagnosis was made by ultrasound imaging and confirmed on tissue samples by a pathologist at the Cancer and Cellular Biological Unit (CCBU) in AL-Najaf Specialized Laboratory in AL-Najaf province, Iraq.

#### **Ethical Approval**

**Valid consent was obtained from each patient before their inclusion in the study according to the Helsinki Declaration-Ethical Principles for Medical Research on Human Subjects. Ethical approval of the sampling procedure was conducted by the Cancer and Cellular Biological Unit in AL-Najaf Specialized Laboratory.**

#### **Tissue dissociation**

Tissues were dissected and incubated in 100µl collagenase (0.25%) for 20 minutes at 37°C. It was vigorously shaken and mechanically digested with a syringe plunger to facilitate cell shedding.

#### **Tissue inhibition**

Using an inhibitor solution (900 µl of RPMI 1640 and 10% fetal bovine serum FBS1), the cells were mechanically digested and washed with 300µl of RPMI 1640 for 5 minutes at 37°C and centrifuged at 1500 RPM for 5 minutes. The supernatant was discarded, and 200µl of RPMI 1640 containing 10% FBS and 1µl Penicillin/Streptomycin solution was added before resuspending and incubating at 37°C for 15 minutes<sup>(18)</sup>.

#### **Tissue growth**

Cells were cultured in 24 non-TC treated plates. Cells were cultured for 7 days and replaced daily. Cells were monitored and photographed using an inverted microscope<sup>(18)</sup>.

#### **Preparation of *A. halimus* extract, Cisplatin, and Combination**

Cisplatin (Cis) and *A. halimus* extract (AHE) were dissolved separately in RPMI 1640 medium and variable concentrations (200, 100, 50, 25, 12.5 µg/ml) were prepared for each. However, a combination (AHC) is a 50% dose of *A. halimus* extract and Cisplatin in the same concentrations.

#### **Cell cytotoxicity determination using MTT assay on human liver carcinoma**

Cells were seeded at 1× 10<sup>5</sup> cells/mL in 96-well microtiter plates in RPMI medium. For 24 hours, the cells were incubated. AHE, Cis, and AHC at various concentrations (200, 100, 50, 25, 12.5 µg/ml) were dissolved in media, and control cells received only serum-free media, added in triplicate, and 24 hours incubation. Following that, the cells were treated for 1 hour with 50µl MTT at a concentration of 2µg/ml<sup>(19)</sup>.

Following the incubation period, the entire contents of the well were aspirated. After incubation at 37°C, 5% of CO<sub>2</sub> and, 50µl of DMSO were applied to each well, and absorbance at 492 nm was measured with a microplate reader<sup>(20)</sup>.

200µL of cell suspensions were seeded at a density of 1 x 10<sup>4</sup> cells mL<sup>-1</sup> in 96-well micro-titration plates and cultured for 48 hours at 37°C to examine cell morphology under an inverted microscope. The medium was then taken out, and 200µL of each concentration of AHE, TAM, and AHT was added. The plates after being stained with 50 L of Crystal Violet and incubated at 37°C for 10-15 minutes after 24 hours, the samples were gently rinsed with tap water until the dye was removed. An inverted microscope was used to examine the cell and calcified the cell growth inhibition rate then photographed with a digital camera<sup>(21)</sup>.

#### **Preparation Sample For Biochemical Test**

Primary cells of human liver carcinoma after the 24 h of treatment, were collected at concentrations (100 µg/ml) from plates culture and transferred to an

empty tube (1.5ml), centrifuged at 13,000 rpm (4-°C) for 10 min, the supernatant was Isolated and stored in deep freezer (-20 C) until assay enzyme activity.

**Superoxide Dismutase (SOD) Activity**

Primary cells of human carcinoma after 24 hours of treatment were collected at concentrations (100 µg/ml) from plates cultured at 13,000 rpm (4°C) for 10 min, and the supernatant was isolated.

SOD activity (Cu-Zn) was measured using a simple and quick approach based on the enzyme's ability to prevent pyrogallol autoxidation. Pyrogallol autoxidation is 50% in the presence of EDTA at pH 8.2. The principle of this method is the struggle between pyrogallol autoxidation by O<sub>2</sub>• and radical dismutation by SOD. SOD activity (Cu-Zn) is measured in milliliters. The amount of enzyme required to inhibit pyrogallol autoxidation by 50% is defined as one unit of (Cu-Zn) SOD activity. Absorption was measured at 420 nm against Tris-EDTA buffer at zero time and after 1 minute of pyrogallol addition <sup>(22,23)</sup>.

**Assessment Catalase Activity**

Primary cells of human carcinoma after 24 hours of treatment were collected at concentrations (100 µg/ml) from plates cultured at 13,000 rpm (4°C) for 10 min, and the supernatant was isolated. The process involves the interaction of ammonium metavanadate with H<sub>2</sub>O<sub>2</sub> under acidic circumstances and is based on the reduction of vanadium(V) to vanadium(III) by H<sub>2</sub>O<sub>2</sub>. Although H<sub>2</sub>O<sub>2</sub> is a strong oxidant, under certain redox conditions it can act as a reductant. As a result, The reduction of vanadium (V) results in the creation of a red-orange peroxovanadium complex with a maximum absorbance of 452 nm. **Hadwan and Abed** <sup>(24)</sup>. Catalase enzyme activity was determined by measuring the absorption of the red-orange peroxovanadium complex at 452 nm. In, the

procedure for enzyme activity was explained (**Table 1**) by <sup>(25)</sup>.

**Table 1. Catalase activity evaluation procedure**

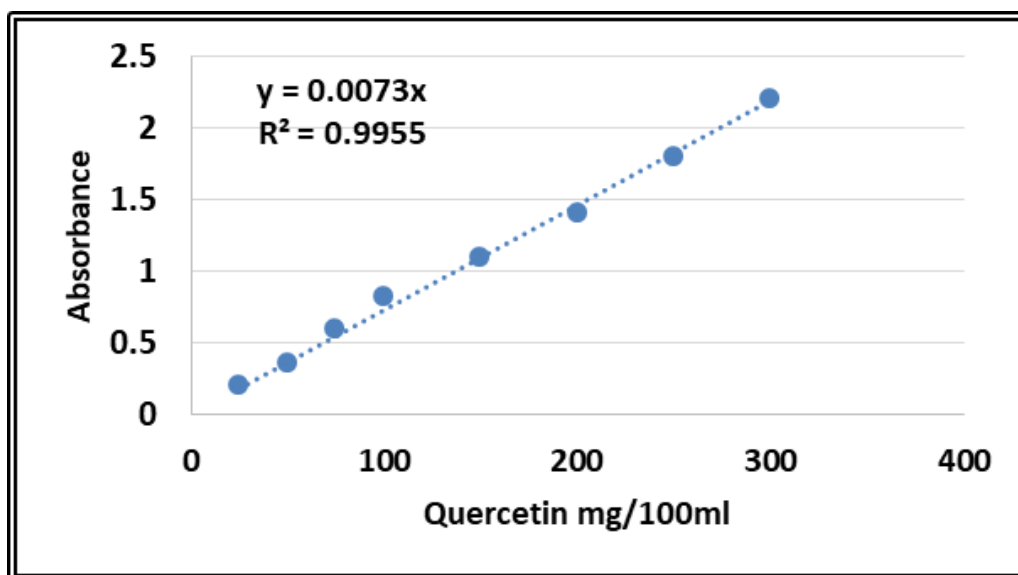
Reagents	Test	Standard	Blank
Sample	1000 µl	-----	-----
Hydrogen peroxide	2000 µl	2000 µl	----
vortex and Incubate at 37 °C for 2 minutes, then add:			
Vanadium reagent	2000 µl		
Distilled water	-----	1000 µl	3000 µl
After that, the tubes were held at 25 °C for 10 minutes. At 452 nm, absorbance changes were observed concerning the reagent blank.			

**Statistical analysis**

Data was collected and analyzed in triplicate for the MTT assay using a graph pad prism, An automated python IC50 calculator was used to calculate (IC50), and for enzyme assay, Paired Samples Statistics were used. Graphs and data were represented using Microsoft Excel 2010.

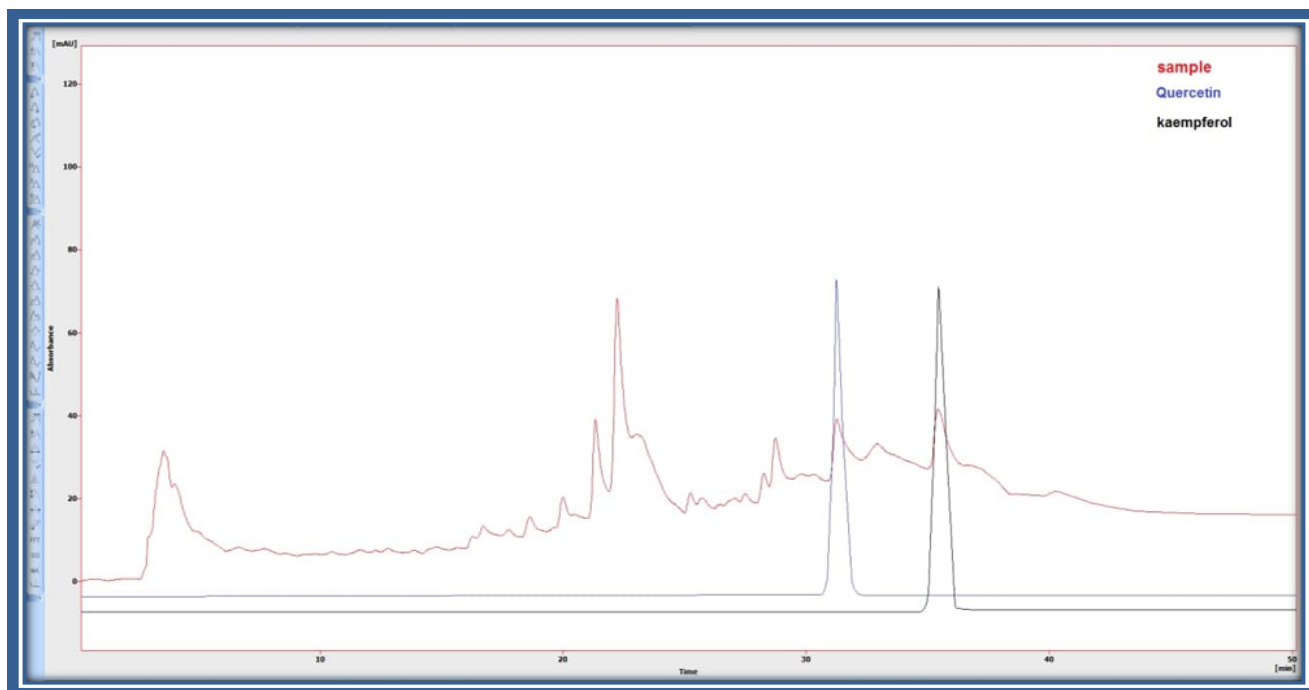
**RESULTS**

Flavonoids have been extracted from the whole plant of *A. halimus* by fractionation of methanol crude extract through the use of different solvents for re-extraction of free (hexane fraction) and bound (ethyl acetate fraction) flavonoids <sup>(26)</sup>. The results showed a total flavonoid yield of 9% w/w. However, the TFC of *A. halimus* was quantified from the calibration curves ( $Y = 0.0073x$ ,  $R^2 = 0.9955$ ) in (**Fig 1**). The results showed that the TFC value was 2.04 mg QE/g in the methanol extract, but in the aqueous extract, it was 1.48 mg QE/g.



**Fig 1. Calibration curve of the quercetin standard solution.**

This is the first study used to identify flavonoids in this species of *A. halimus* by HPLC that uses retention time and UV/vis spectrum matching with its respected standard material to quantify flavonoids compounds. Quercetin and kaempferol were identified by HPLC chromatogram (**Fig 2**).



**Fig 2. HPLC Chromatogram of *A. halimus* extract**

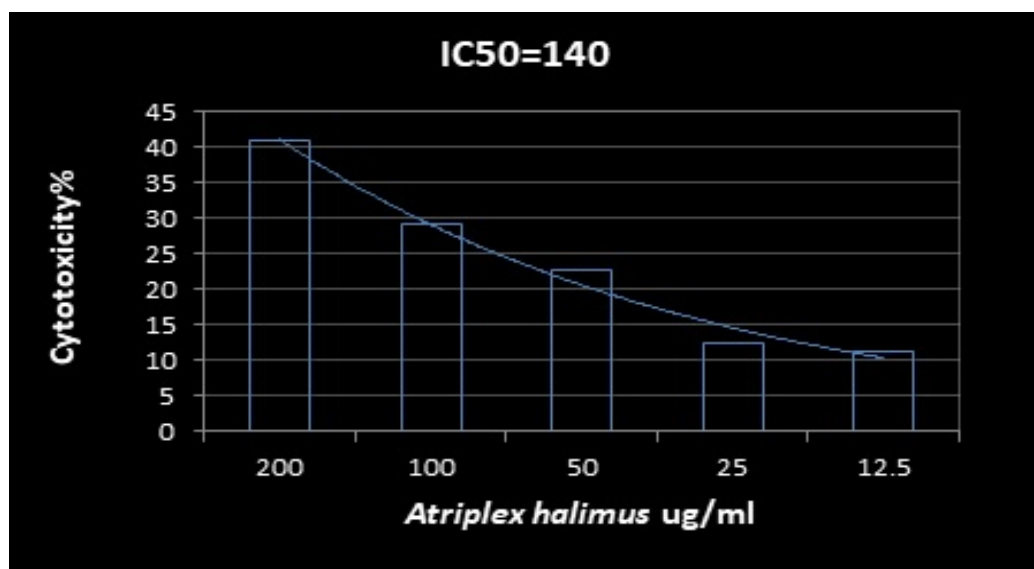
A the concentration of quercetin and kaempferol was (8.5387941 and 7.6326572  $\mu\text{g/ml}$ ) (**Table 2**).

**Table 2. Identified qualitative and quantity of flavonoids in *A. halimus* Extract**

Sample	peak area	$\mu\text{g/ml}$	total $\mu\text{g}$	plant weight $\mu\text{g/mg}$
Quercetin	340.191	8.5387941	25.6163823	0.256163823
Kaempferol	360.031	7.6326572	22.8979716	0.228979716

The anticancer activity of AHE was tested on cancer cells and compared with cisplatin as well as the combination to produce a new treatment and assess the synergistic or antagonistic effects on cells. The results from the MTT assay of AHE, Cis, and AHC show the highest cytotoxicity on carcinoma cell growth inhibition at concentrations (100 and 200  $\mu\text{g/ml}$ ) and were (29.14 and 40.97%) (**Fig 3**), (46.15 and 64.66%) (**Fig 4**) and (38.54 and 61.32 %) (**Fig 5**), respectively.

The IC<sub>50</sub> value of this assay for AHE, Cis, and AHC was (140, 110, and 111.4  $\mu\text{g/ml}$ ), respectively.



**Fig 3. A graphical illustration of AHE's cytotoxic effect on cancer cells.**

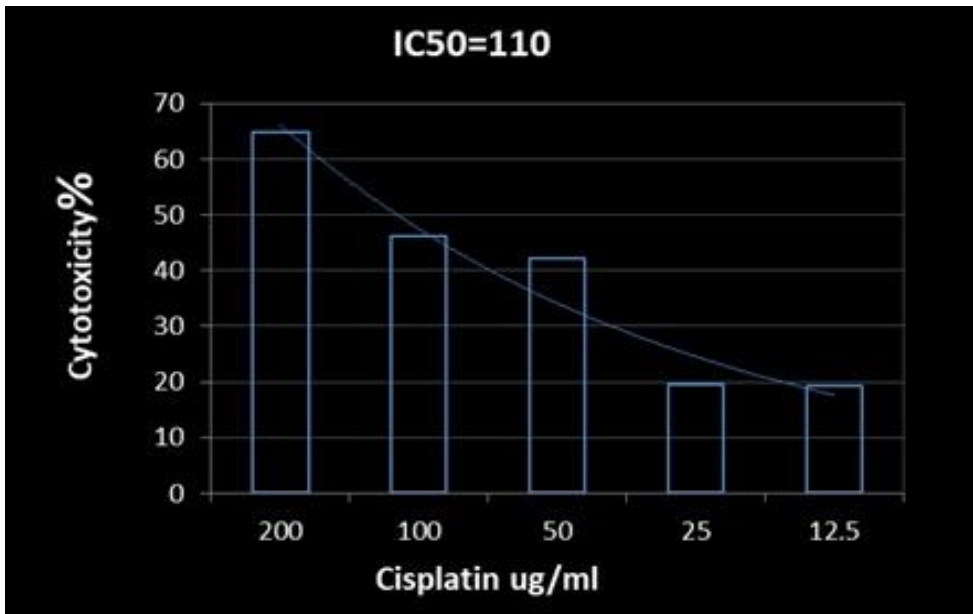


Fig 4. A graphical illustration of Cis cytotoxic effect on cancer cells.

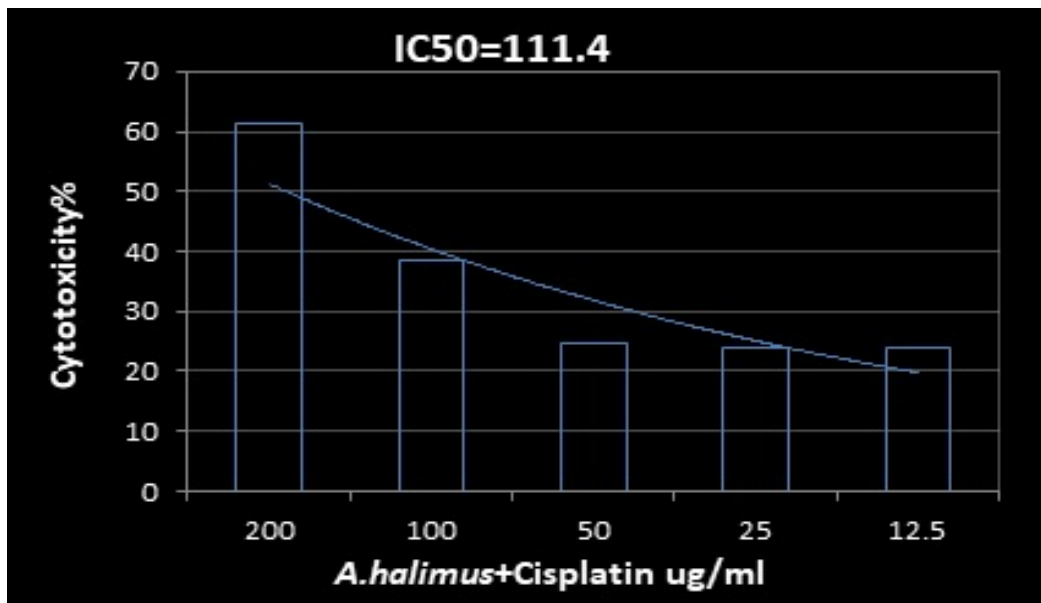


Fig 5. A graphical illustration of AHC cytotoxic effect on cancer cells.

The results from IC50 values indicate Cis was the most effective concentration compared to AHE and AHC, whereas the combined shows the highest cytotoxicity effect than AHE. The level of SOD and catalase enzymes was measured on the cancer cell. Cancer cells were exposed to *A. halimus* extract, cisplatin, and combination at a concentration of (100 ug/ml) for 24 hours and as a control, untreated cells were employed. The results were shown in (Table 3) and appeared significant differences in increased SOD level (P = 0.001) (Fig 6) and a decreased catalase level (P=0.032) (Fig 7) on cancer cells exposed to these treatments.

Table 3. Effect of *A. halimus* extract, Cis, and Combination on SOD and Catalase activity in Cancer Cells

Enzyme activity	Control	<i>A. halimus</i>	Chemotherapy drug	Combination
SOD (U/ ml)	1.888	5.823	6.888	5.703
Catalase (Katal /ml)	1.696	1.110	1.337	1.012
Breast	1.797	1.84	1.828	1.835

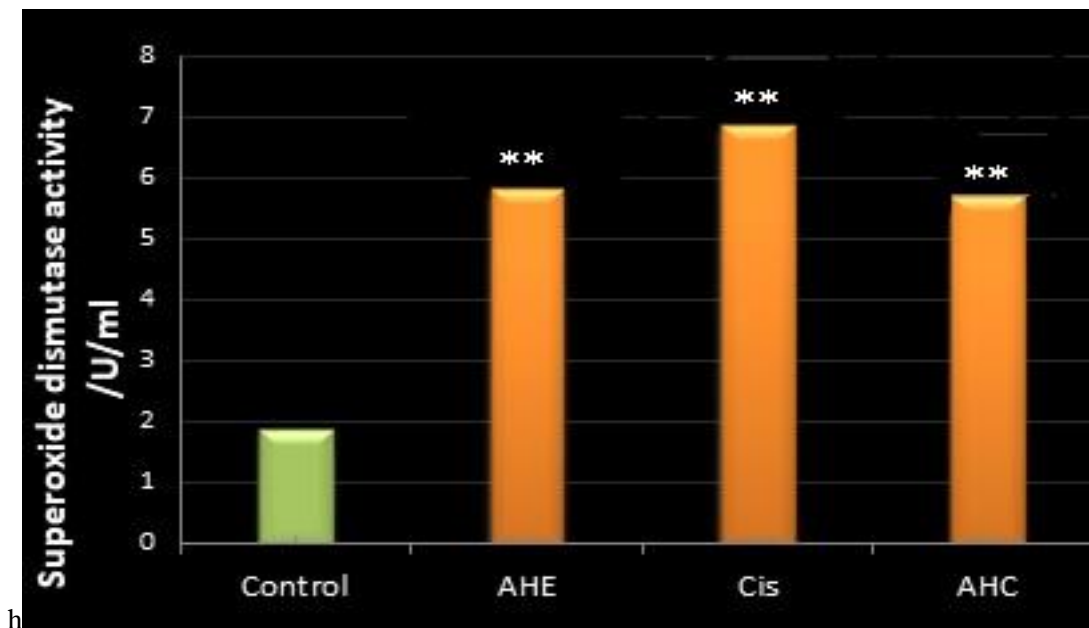


Fig 6. Effect of AHE, Cis, and AHC on SOD activity in Cancer cells (p-value <0.05).

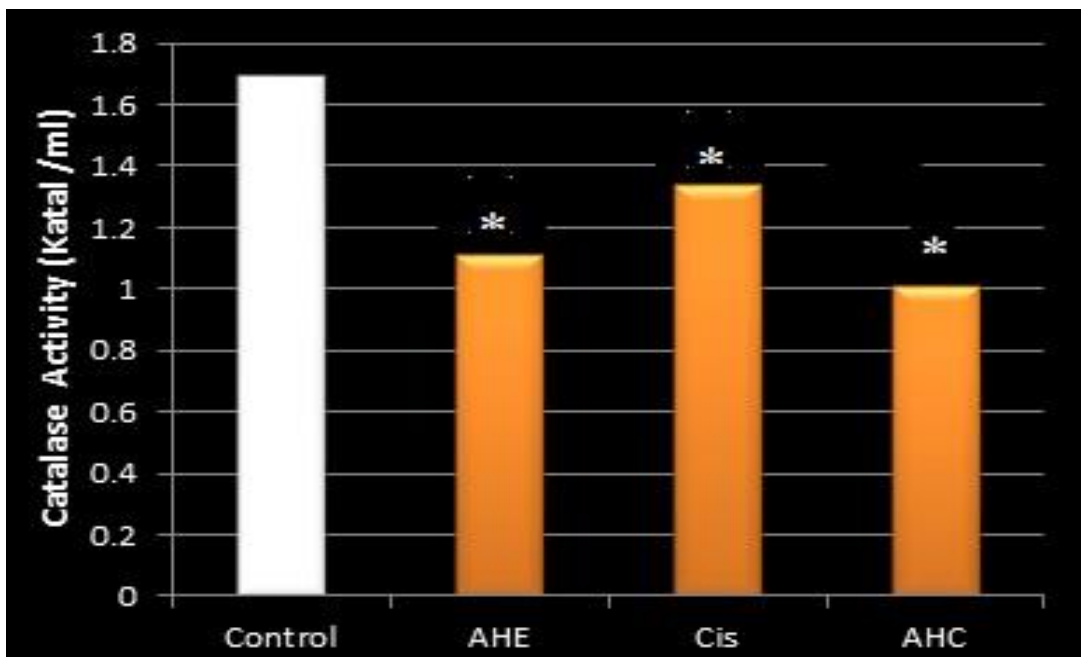
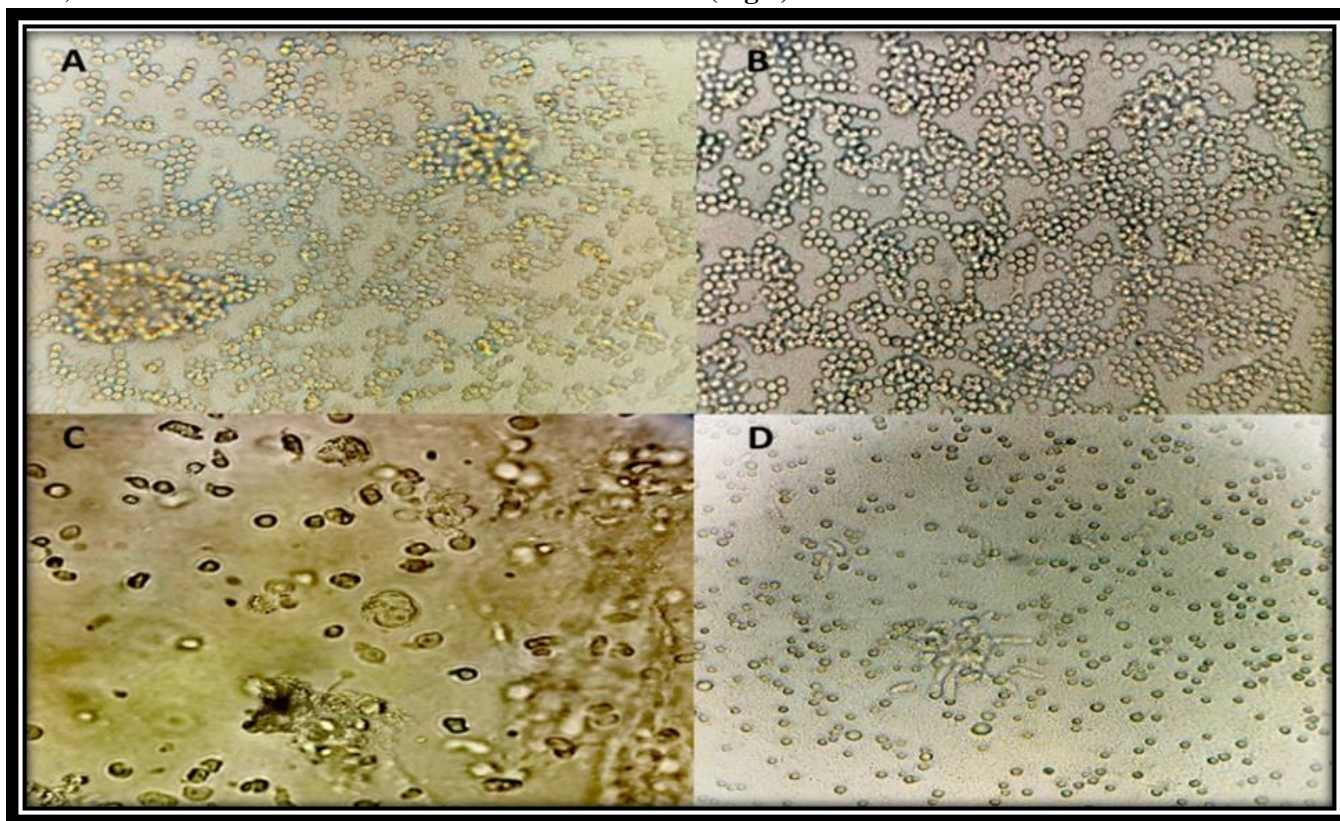


Fig 7. Effect of AHE, Cis, and AHT on catalase activity in cancer cells (p-value<0.05)



In addition, Advanced imaging techniques showed Cis a higher effect on the reduced proliferation of cancer cells, while the effect of the AHE was lower than the AHC (Fig 8).



**Fig 8. Morphological Appearance of Human Liver carcinoma**

(A) Normal cancer cell morphology was seen under the control condition, X 400. (B) Liver cancer cells were seen to have moderate cell morphology including undefined cell boundaries when exposed to *A. halimus* extract, X4000.(C) Apparent morphological and cell apoptotic changes were seen in liver cancer cells when exposed to cisplatin including cell pigmentation and shrinking, X1000.(D) Moderate cell morphology with cell apoptosis is seen when liver cancer cells were exposed to combination, X400.

## DISCUSSION

Natural medicines have attracted a lot of attention in the treatment of cancer because of their safety, efficacy, and lack of side effects **Wang *et al.*** <sup>(27)</sup>. Flavonoids are exploited commercially as biologically active chemicals and typically high-value-low volume products, and the highest value of flavonoids are found in methanol extracts compared with aqueous extracts and this agrees with **Kheira *et al.*** <sup>(28)</sup>. The difference in polarity of the organic solvent's effect on the isolated phytochemical molecule determines the variation of TFC in the extracts.

However, the identification of these compounds in *A. halimus* is of great importance from a medical point of view. Therefore, the HPLC technique was used in diagnosing and determining their concentrations by using a variety of standards. Quercetin and kaempferol consider natural antioxidants and could help us to understand the action of plant behaviors in biological functions and to discover new targets <sup>(29)</sup>.

According to this study, **Davis and Matthew** <sup>(30)</sup> recorded that quercetin has growth-inhibiting effects on various malignant tumor cell lines in vitro. These included P-388 leukemia cells, colon cancer cells, human breast cancer cells, and human squamous as well as ovarian cancer cells. While another study found that kaempferol suppressed HepG2 cell proliferation, migration, and invasion by upregulating PTEN and downregulating miR-21 in addition to deactivating the PI3K/AKT/mTOR signaling pathway <sup>(31)</sup>.

Cisplatin showed the highest cytotoxicity on liver cancer cells and other forms of malignancies, such as leukemia, lymphomas, breast, testicular, ovarian, and sarcomas, where they are treated with cisplatin as the first-line chemotherapy drug. Once within the cell, cisplatin begins to cause cytotoxicity by losing one chloride ligand, attaching to DNA to create intra-strand DNA adducts, and preventing DNA synthesis and cell development. By stopping cisplatin-induced cell death by activating the ATM (ataxia telangiectasia mutated) pathway, the DNA lesions created by

cisplatin-induced DNA damage stimulate DNA repair response via NER (nuclear excision repair system) <sup>(32)</sup>.

At the same time, according to the present study, the cytotoxic effect of *A. halimus*-induced cancer cell death may be caused by the generation of intracellular ROS, which results in altered mitochondrial function and caspase 3 activations. The cytotoxic effect of *A. halimus* on cancer cells can be increased by an increased dose of AHE due to the absence of side effects of this plant **Benarba** <sup>(33)</sup>. In contrast to most chemotherapy drugs like cisplatin that may be causes nephrotoxicity, hepatotoxicity, and neurotoxicity <sup>(34)</sup>.

However, AHC shows better efficacy than AHE, this combination gives a synergistic effect. At the same time, AHC can be useful in reducing the side effects of cisplatin because it contains powerful antioxidants and maintains the efficiency of chemotherapy, as shown in this previous study, which indicates that combination therapy with cisplatin and natural products is effective in reducing resistance to cisplatin therapy <sup>(34)</sup>.

Low SOD activities in several malignancies suggest reduced ROS protection **Xu et al.** <sup>(23)</sup> This proves that low levels of the SOD enzyme in cancer cells could be supplemented by exogenous antioxidants delivered by exposure to *A. halimus* extract, which has potent antioxidants. The same effect was given on cancer cells when compared with chemotherapeutic drugs and was better than combined. Increased SOD enzymes can serve as tumor suppressor genes for these cancer cells, and these genes are specialized proteins tasked with inducing apoptosis and DNA repair **Fonseca et al.** <sup>(35)</sup>. Apoptosis can be initiated through two pathways, the extrinsic or the intrinsic (mitochondrial) pathway while superoxide dismutase is a primary antioxidant enzyme found in the mitochondria which are also mediating the intrinsic pathway of apoptosis. this apoptosis shows obviously in liver cancer cells when exposed to a concentrated dose of treatments and monitored by microscope during an MTT assay.

Catalase is one of the essential antioxidant enzymes that play a critical regulator of the metabolism of hydrogen peroxide. The result showed that catalase enzyme levels are high in the control cancer cells hence acquiring resistance against oxidative stress. While, after exposure of these cancer cells to various treatments, there was a decrease in the levels of catalase enzyme and significant differences between treated and untreated cells as shown in the previous study **Fonseca et al.** <sup>(35)</sup> This decrease in the levels of catalase enzyme in the treated cells leads to an increase in oxidative stress, which in turn leads to cell death by apoptosis. So obviously *A. halimus* extract has potent antioxidants and gives the same effect on cancer cells when compared with chemotherapeutic drugs and combination.

Cisplatin is an effective chemotherapy drug that can be used to treat a variety of solid tumors,

including cancer. Cisplatin can also cause liver damage when given in high doses, and its mechanism may entail an increase in the formation of ROS and oxidative stress according to this studies .

While the effect of the AHC was higher effect than *A. halimus* due to make a synergistic effect on cancer cells compared with AHE alone. this *A. halimus* content potent antioxidant of flavonoids like quercetin and kaempferol and this is useful by inducing apoptosis in cancer cells and reversal of multidrug resistance by working as an inducer that increases the generation of ROS to the point where it crosses the toxic threshold, activating apoptosis. Mrf2 and GSH are important regulators of the equilibrium of ROS in cells, and certain flavonoids can limit their activity to produce re-sensitizing effects .

Additionally, a previous study showed that flavonoids can be employed in chemotherapy due to both their ability to fight tumors and the increased cytotoxic effects of chemotherapeutic drugs . As a result, this will reduce the dose of chemotherapy and have the same effect on the growth and proliferation of cancer cells.

## CONCLUSION

*A. halimus* extracts exhibit anticancer effects in the primary cell culture of human liver carcinoma chosen in this study with morphological alterations that help apoptosis be induced. Also, the extract of *A. halimus* showed potent antioxidant activity by measuring superoxide dismutase and catalase enzymes. This plant extract can be considered a potential candidate for use in pharmaceutical products. Consequently of having a combination of quercetin and kaempferol in addition to other bioactive compounds.

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- **Authors' contributions**
- The final manuscript has been reviewed and approved by all authors.
- **Competing interests**
- The authors declare to have no conflicts of interest.

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