



## Green plant *Laurus nobilis* L. leaves extract hybrid material as a potential chemotherapy sensitizer for the treatment of hepatocellular carcinoma

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### ABSTRACT

Background: Cisplatin is considered a highly successful chemotherapy medication in treating most solid tumors with worldwide with severe side effects. So, the aim of this study is to overcome cisplatin side effects by hybrid with *Laurus nobilis* L. leaves extract as a potential chemotherapy-sensitizer for the treatment of hepatocellular carcinoma. Methods: We make this hybrid by drying, grinding bay leaves and boiling them with distilled water and filtered, then its suspension added to the equal volume of cisplatin, characterize this hybrid compound by cytotoxicity test and chromatography to evaluate its antioxidant, antimicrobial, and anti-inflammatory bioactivities, and investigate a hybrid compound. Anti-cancer effects were estimated in HepG-2 cell line. Results: The cell proliferation test was used to measure metabolic processes leading to cell apoptosis that identify cell viability against hepatocellular carcinoma with  $IC_{50} = 0.08 \pm 0.01 \mu\text{g/ml}$  under these experimental conditions and good results in chromatography test has phenolic compounds and essential oils have antioxidant properties with a surface which was acidic ( $KD/KA=0.95$ ). Conclusions: cisplatin in combination with *Laurus nobilis* L. leaf extract can be used as a promising regimen in treating hepatocellular carcinoma.

**Keywords:** Chemotherapy, Cisplatin, Hepatocellular carcinoma, Hybrid compound, *Laurus nobilis* L. leaves

### 1. INTRODUCTION

The liver is important for vertebrates and other animals as it has many functions in the body such as metabolism, immunity, synthesis of proteins, detoxification and aids in digestion [1]. It is susceptible to a lot of diseases; most common of these diseases are hepatitis A, B, C, D and E causing liver inflammation that followed with liver fibrosis, cirrhosis and hepatocellular carcinoma [2].

Hepatocellular carcinoma (HCC) still a dangerous alarm bell and its prevalence is growing worldwide as it is the most common form of liver cancer related morbidity and mortality in the world [3]. The Barcelona Clinic Liver Cancer (BCLC) and American Joint Committee on Cancer (AJCC) Tumor Node-

Metastasis (TNM) are the two stages of HCC treatment. While the second based on the tumor features, patient performance status and severity of the liver disease [4]. Several cytotoxic chemotherapeutic drugs are used in liver cancer treatment as single systemic therapies, such as cisplatin, doxorubicin, 5-fluorouracil, or a combined regimen but all of them had three disadvantages: (1) Their response rate was 10 - 25% with little improvement in survival rate, (2) patients with underlying cirrhosis don't tolerate these treatments well, and (3) HCC is highly resistant to a single treatment system [5].

Cisplatin is considered one of the most successful drugs worldwide used in chemotherapy [6]. It was accidentally discovered in 1965 by Barnett Rosenberg and has been known as an anticancer medication from this time. This treatment is a platinum compound with a square planar coordination with two chlorides and two group of ammonia in the cis structure [7]. Its formula  $[Pt(NH_3)_2Cl_2]$ .

Although the high success of cisplatin in treating hepatocellular carcinoma (HCC), severe side effects limit its use like nephrotoxicity, peripheral neuropathy, severe nausea, vomiting, temporary hair loss, loss in the ability to taste food, dry mouth, Hiccups and myelosuppression so there is a need for hybrid another organic compounds extract with cisplatin to overcome its side effects as a potential chemotherapy-sensitizer for the treatment of HCC [8].

*Laurus nobilis* L. which is also known as bay is a plant species from Lauraceae family [9]; Iranian folk medicine used the leaves of *Laurus nobilis* in treating many diseases like nervous system; Also traditionally used orally in treating the problems of stomach and colon, such as epigastria bloating and flatulence, and can also be used to treat cancer [10].

Leaves are bioactive compounds contain terpenes and its derivatives, alkaloids, minerals and vitamin, polyphenols [11], programmed apoptotic leukemia cells (HL-60) induced by an isolated derivatives from *Laurus nobilis* leaves show potential as a hybrid compound for development of new classes of antileukemic drug [12].

In this regard, the current study aims to create a hybrid compound with anti-cancerous and biocompatible qualities. This hybrid compound was created to overcome the disadvantages of utilizing anticancer medicines on their own. Cisplatin, the most commonly used chemotherapy medication, was grafted *Laurus nobilis* L. leaves extracts and tested for anticancer efficacy on a liver cancer cell line.

## 2. MATERIALS AND METHODS

**2.1. Chemicals:** Cisplatin ( $cis-PtCl_2(NH_3)_2$ ) (code: 5622539) with concentration 1mg/mL was obtained from Oncotec Pharma Production GmbH.

**2.1.2. *Laurus nobilis* L. leaves extraction:** We purchased Bay leaves from traditional herbal markets. The leaves were washed, dried in the shade and ground to powder by electric grinder. The crude extracts were prepared by adding about 20 grams of powder of the leaves to 100 ml of distilled water in a conical flask. Then the mixture was boiled for 10 minutes and cooled to 37°C. Then the crude extracts were filtered and dried using Lyophilized. The extracts were prepared following Rizwana et al. method [13].

**2.1.3. Preparation of Cisplatin hybrid compound:** 20 ml of Cisplatin (1mg/ml) were added to the equal volume of *Laurus nobilis* L. leaves suspension prepared, with continuous stirring for two hours. Then store this hybrid compound in a clean sealed bottle.

**2.1.4. Characterizations of Cisplatin hybrid compound:** The Cisplatin hybrid compound prepared before and after surface modifications were characterized by their particle size, and surface charge. Reverse phase gas chromatography was used to identify surface properties bay leaves. From this, it was found that the leaf surface of plant leaves is acidic ( $KD/K_A=0.95$ ).

**2.1.5. Analysis of bay leaves component by Gas chromatography test:** The chromatography test was done in Ain Shams University. A Perkin Elmer Clarus 500 GC gas chromatograph combined with a Perkin Elmer Clarus 560 mass spectrometer was used for the GC-MS analysis to identify the major components of the essential oils of bay leaves. The chromatographic separation was performed by Perkin

Elmer Elite-5 fused-silica capillary column (30 m × 0.25 mm, film thickness 0.25 μm). Then increase the temperature of the column by 4 °C every minute from 50 °C for 5 min to 280 °C. A carrier gas used at a constant flow rate of 1 mL/min was helium. Temperature of the oven was 250°C [14].

**2.1.6. Encapsulation Efficiency and Loading capacity:** Encapsulation efficiency is an expression of the amount of cisplatin incorporated into the *Laurus nobilis* L. leaves suspension prepared. It is usually defined as the percentage of cisplatin bound to *Laurus nobilis* L. leaves suspension prepared relative to the total amount of cisplatin used. Determination of this parameter generally requires analysis of free and encapsulated cisplatin fractions on the *Laurus nobilis* L. leaves suspension prepared allowing calculation of encapsulation efficiency. The efficiency of cisplatin encapsulation and loading capacity (LC) of the process were expressed as a percentage of encapsulated drug and were calculated using the following formulas (1) and (2) shown below:

$$\text{encapsulation \%} = \frac{\text{Cisplatin total} - \text{Cisplatin Free}}{\text{Cisplatin total}} \times 100$$

$$\text{Loading capacity \%} = \frac{\text{Cisplatin total} - \text{Cisplatin free}}{\text{hybrid compound weight}} \times 100$$

### 2.1.7. Cell culture study

**2.1.7. a. Mammalian cell lines:** The human hepatocellular cancer cell line (HepG-2 cells) was getting from the American Type Culture Collection (ATCC, Rockville, MD).

**2.1.7. b. Reagents:** the reagents used were getting from Sigma company located in Saint Louis, in the American United states which were dimethyl sulfoxide (DMSO), 3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and trypan blue dye but from Lonza company located in Belgium, we got cell culture medium called Dulbecco's Modified Eagle, Fetal Bovine serum, a zwitterionic organic chemical buffering agent, other reagents used for cell culture. All reagents applied at different concentrations.

### 2.1.8. Calculation of Cytotoxic Effects

**2.1.8. a. Cell lineage spread:** This test was done in the Biotechnology Center, Damietta University. Cells were cultured in RPMI-1640 medium supplemented with 10% inactivated fetal bovine serum and 50 μg/ml gentamycin. Cells were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere and cultured two to three times.

**2.1.8. b. Cytotoxicity assessment using Viability test:** For antitumor experiments, cancer cells were suspended in medium at concentration of 5 × 10<sup>4</sup> cell/well in Corning® 96-well tissue culture plates, and then 24 hour incubation. The tested hybrid compounds were put into 96-well plates (three times) to achieve twelve concentrations for each compound. Six vehicle controls with media or 0.5 % DMSO were run per each 96 well plate as a control; the numbers of viable cells were determined by cell cytotoxicity test after incubating for twenty four hour. Briefly, removing media from 96 wells and replaced with 100 μl of fresh medium then 10 μl of the 12 mM stock solution (5 mg of MTT in 1 mL of PBS) per well including the untreated controls. ELISA plate was put at 25°C and carbon dioxide five percent for 240 minutes. An eighty five μl portion of modified Eagle medium was removed. For ten minutes; 50 μl of previous medium were added to each sample, mixing by the pipette and put in incubator. After that, measuring at 590 nm using microplate reader getting from Sunrise from the American United states to assess the cell viability percentage by [(optical density<sub>treated</sub>/ optical density<sub>untreated</sub>)] × 100% where the optical density for each parameter is the average of its parameter. Also, cytotoxicity test used to assess IC<sub>50</sub> which is the concentration giving the toxicity in fifty percentages of healthy cells [15, 16].

## 3. RESULTS AND DISCUSSION

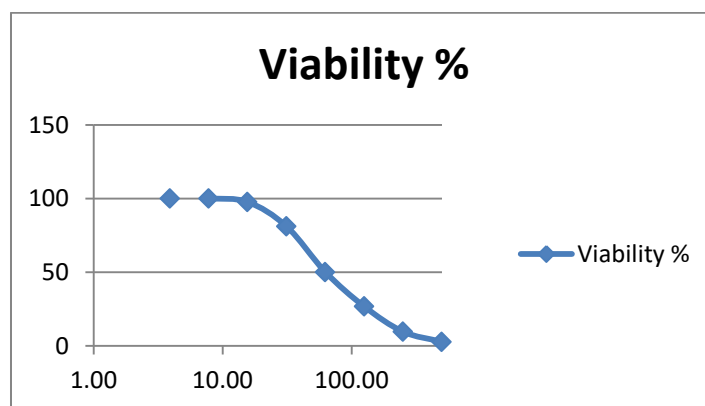
### In vitro examinations

The antitumor activity of hybrid compound was evaluated using HepG-2 cell lines. The effect of *Laurus nobilis* L. leaves on HepG-2 cell lines was evaluating in various concentrations and data were shown in table 1 and graphically in Figure 1 with IC<sub>50</sub> = 67.83 ± 13.53 μg/ml. Table 2 and Figure 2 shows

that normal cell cytotoxicity from the laurus nonilis l. Aq. Extract with cisplatin ( $IC_{50} = 93.7 \pm 4.78$ ) while table 3 and Figure 3 show that the cytotoxicity against HepG-2 cell line by Laurus nobilis l. Aq. extract with cisplatin ( $IC_{50} = 0.08 \pm 0.01 \mu\text{g/ml}$ ). The present study shows that the hybrid compound has excellent anti-growth capabilities and is superior to cisplatin in hepatocellular carcinoma. The HepG-2 cell line demonstrated that the hybrid compound delivery systems can increase cytotoxicity in vitro.

**Table 1: Cytotoxicity assessment Laurus nonilis aq. extract against HepG-2 cell by sample:**

Sample conc. ( $\mu\text{g/ml}$ )	Viability %	Inhibitory %
500	2.81	97.25
250	9.68	90.32
125	26.77	72.86
62.5	50.13	50.48
31.25	81.11	19.21
15.6	97.67	1.87
7.8	100	0
3.9	100	0
0	100	0



**Figure1: assessment of inhibitory activity against liver cancer cells with  $IC_{50} = 67.83 \pm 13.53 \mu\text{g/ml}$ .**

**Table 2: Assessment of cytotoxicity of Laurus nonilis aq. extract with cisplatin against normal cell by sample:**

Sample conc. ( $\mu\text{g/ml}$ )	Viability %	Inhibitory %
500	5.97	92.87
250	12.65	87.67
125	39.14	66.15
62.5	77.98	26.81
31.25	95.3	4.87
15.6	97.12	0.48
7.8	100	0
3.9	100	0
0	100	0

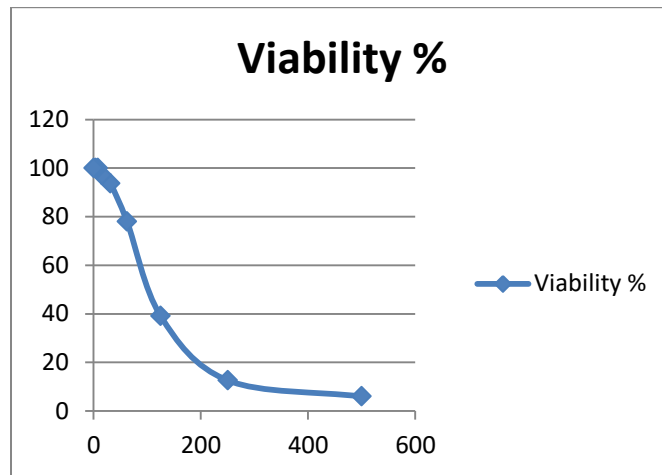


Figure 2: Inhibitory activity against normal cell line with IC<sub>50</sub> = 93.7 ± 4.7 µg/ml.

Table 3: Assessment of IC<sub>50</sub> of Laurus nobilis aq. extract with cisplatin against cancer cells by sample:

Sample conc. (µg/ml)	Viability %	Inhibitory %
500	1.2	0.01
250	1.0	0.06
125	0.8	1.18
62.5	0.6	24.3
31.25	0.4	71.5
15.6	0.17	80.4
7.8	0.09	95.4
3.9	0	100
0	0	100

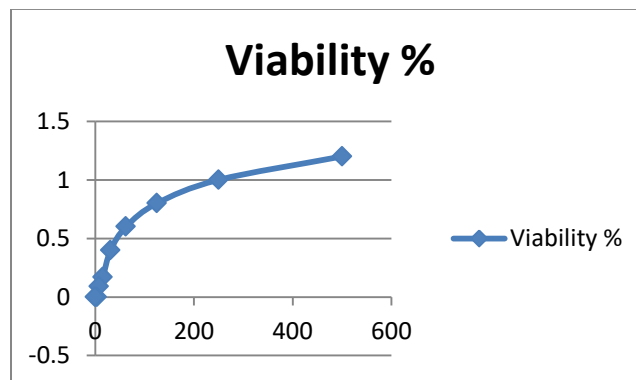
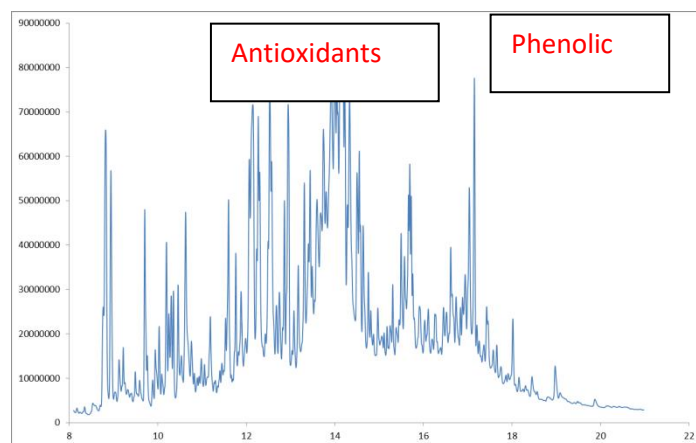


Figure 3: Inhibitory activity against Hepatocellular carcinoma cells with IC<sub>50</sub> = 0.08 ± 0.01 µg/ml.

In chromatography test, we found that Laurus nobilis L. leaves contain aromatic essential oils which evaluate its biological activities like; antioxidant, antidiabetic, antiobesity, antimicrobial, and antimutagenic. The results show that about 99% of the total oil consisted of 51 compounds, aromatic essential oil is one of these compound that its metabolites were characteristic with many oxidized

terpenoids with 1,8-Cineole and Terpinyl acetate, 3TMS derivative,  $\beta$ -pinenes, Glycerol, Palmitic acid and etc. (Figure4).



**Figure 4: Chromatography result of *Laurus nobilis* L. leaves**

The traditional chemotherapeutic medications used to treat a wide range of solid tumors have obstacles due to their side effects and their toxicity on normal cells. So, there is a continuous search to make hybrid compounds with these chemotherapeutic medications to avoid their side effects through improved penetrability and retention effect (EPR) for passive targeting.

Medicinal plants are used all over the world to treat a lot of diseases. as the treatment of diseases with the traditional herbal has no side effects and safe in use but modern synthetic pharmaceuticals and the simultaneous have bad side effects and they increase in multi-drug resistant microbes [16].

During the last decade, interest in phenolic compounds has increased due to their biological effects and therapeutic applications as they play a vital role in human health through their activities as anti-allergic, decrease or removes inflammatory, kill microbes and anti-cancers [17].

Traditionally, bay leaves used orally to treat of gastrointestinal symptoms problems due to the bay leaves and fruits have aromatic, narcotic and stimulant properties. [18].

The chromatography results indicate that *Laurus nobilis* l. leaves have Phenolics , Terpenoids, essential oils with highly concentrations that have antioxidants and anti-inflammatory properties. It has been reported that the plants main sesquiterpene lactone, costunolide, and its alpha-methylene-gamma-butyrolactone molecules, are important in this activity. The antioxidant activity of *L. nobilis* leaves was studied, and it was found that the compound responsible for the alkyl radical scavenging activity was isoquercitrin. The sesquiterpenes costunolide and zaluzanin D get from bay leaves showed potent growth inhibitory effects against human myelogenous leukemia (HL-60) cells [19].

#### 4. CONCLUSION

The hybrid compound (*Laurus nobilis* L. leaves extract with cisplatin) could become an effective chemotherapeutic method for hepatocellular carcinoma and could be a superior candidate for drugs invention. It showed a profile of an impressive biocompatibility represents its suitability targeting for cancer cells. It can be deposited in cancer cells and high their permeability and concentration thus reducing the dose of drug that required to give a higher anti-tumor effect and reduce its toxic.

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