Study the Effect of *Ferula hermonis* Extract on HepG2 cell line culture

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Abstract

Ferula hermonis Boiss (Apiaceae) is known in the Midleast as "zallouh". In the last decade, scientists paid attention to study chemistry and biological activity of the plant. In this study *Ferula hermonis* were collected and evaluated for cytotoxic activity. The plant was extracted with methanol The cytotoxic effect of the extracts was examined in-vitro on human cell line (Human liver carcinoma cell line HepG2). Results were shown that significant decrease of HepG2 cells viability. The effect of *F. hermonis* on cell viability of HepG2 cell line culture show that plant extract cause significant decrease ($p < 0.05$) of HepG2 cell viability, and this decrease in cell viability is increased with the increasing the extract concentrations in comparison with the negative control, and the growth inhibition percentage reach to (78.3%).

Introduction

Ferula hermonis Boiss. (Apiaceae), commonly known as " Shilsh-el-zallouh" , "Hashishat-al-kattira" or "The Lebanese Viagra" ,is a small perennial shrub that grows abundantly at more than 2500 m on the high mountain areas of northern Lebanon, and on the biblical Mount Hermon in Southern Lebanon, (El-Taher *et al*., 2001). Middle East herbalists have used the seeds and roots of this plant for centuries as a folk remedy as an aphrodisiac to treat frigidity in women, and erectile and sexual dysfunction in men by increasing blood flow to sexual organs. The antimicrobial activity of the crude extract and the isolated compounds has been reported (Hilan *et al*., 2007). Previous phytochemical studies on this plant have revealed the presence of various sesquiterpenes, mainly of the daucane ester type (Galal *et al.,* 2001). On roots of *Ferula hermonis,* several sesquiterpenes have been isolated, most of which display biological activities. The active constituents are believed to be daucane alcohols

esterified with aromatic acids, as the compound ferutinol p-hydroxybenzoate (ferutinine) (Ibraheim *et al.*, 2012). Other sesquiterpenes like ferutinol benzoate (teferdin) and ferutinol vanillate (teferin) found in roots. Ferutinine and tenuferidine have demonstrated estrogenic activity. Roots of *Ferula hermonis* also contain triterpenoids, saponins, alkaloids, tannins, steroids mainly neutral lipids and flavonoids as well as vitamins A, B1,B2, B6, C, D and E. The minerals magnesium, selenium, zinc and iron have been identified in the roots (Shavit, 2009).

Materials and Methods

Solutions preparation

RPMI-1640 Medium

This medium contained the RPMI-1640 medium base 10gm, Fetal bovine serum 10% , Penicillin 1000000IU, Streptomycin 1g , Heps 4g, Sodium bicarbonate 1% and Brdu 1%.

Fetal calf serum (FCS)

Aliquot of 10% FCS added to the media to support the cell growth.

phosphate buffer saline (PBS)

One tablet of PBS was dissolved in 200 ml of distilled water sterilized by autoclave then used.

Antibiotic solution

Two antibiotics were used; they were penicillin and streptomycin. The penicillin, sodium salt (1000000 IU) and streptomycin (1 g) were each dissolved in 10 ml of distilled water (D.W) and stored at -20ºC from each of these stocks 0.5 ml was added to one liter of culture media (Freshney, 2000).

Neutral red dye

A quantity of 10mg of neutral red was dissolved in 100 ml of PBS, mixed thoroughly and used immediately (Abdul-Majeed, 2000).

Elution Buffer

It was freshly prepared by mixing phosphate buffer saline to absolute ethanol (v/v) then used directly.

Trypan blue stain

This stain was prepared by dissolving 0.1 g of trypan blue stain in 100 ml of phosphate buffer saline, then flirtation using filter paper. Finally the solution was stored at 4ºC until used (Freshney, 2000).

Trypsin solution

Trypsin 2.5 g for each 100 ml of PBS dissolved then sterilized by filtering using 0.22 µm Millipore filter and stored at 4ºC (Freshney, 2000).

Trypsin – Versene Solution

It was freshly prepared by mixing 20 ml of trypsin solution, 10 ml of versene solution and 370 ml phosphate buffer saline and kept at 4°C until use.

Versene Solution

Versene solution was prepared by dissolving 1g of ethylene-diamine-tetraacetic acid (EDTA) in 100ml of phosphate buffer saline, then sterilized by autoclaving and stored at 4°C.

Cell Line

The Hep G2 cell line (human liver carcinoma cell line) was kindly provided by Animal cell culture laboratory, Biotechnology Research Center / AL- Nahrain University.

The Plant *Ferula hermonis*

Ferula hermonis was obtained from a local market in Baghdad (Alshowrja) and further identified by Dr. Ali Al- Mosawy (Department of Biology, College of Science, University of Baghdad). The plant roots were air dried at room temperature and grinded into powder form.

Plant Extract Preparation of

The dried roots of the plant was powdered using a blinder for five minutes, and then extracted with absolute methanol,50 grams of the processed plant were extracted in 250 ml of the solvent and left in shaking water path (40ºC) for 24hrs. Extract was then filtered with gauze and then with filter paper. The obtained extract was then evaporated at (45ºC) using a rotary evaporator.

Chemical Detection of Plant Extracts

The chemical detection of the plant methanol extract was carried out at the Biotechnology Research Center (AI-Nahrain University).

✸Detection of Terpenes and Steroid

Aliquot of 1 ml of methanolic extract was participated in a few drops of chloroform , then a drop of acetic anhydride and a drop of concentrated sulphuric acid were added, brown precipitate appeared which representing the presence of terpene , which the appearance of dark blue color after few minutes would represent the presence of steroids (Al-Abid, 1985).

✸Detection of Flavonoids

Roots methanol extract of the plant was partitioned (Separatory funnel) with petroleum ether, the aqueous layer was mixed with the ammonia solution. The appearance of dark green color was an indication for the presence of flavonoids (Harborne,1973).

✸Detection of Resins

Aliquot of 10 ml of distilled water acidified with 4% hydrochloric acid were added to 5 ml of the plant extract, the appearance of turbidity indicated the presence of resins (Shihata, 1951).

✸Detection of Tannins

Aliquot of (25ml) of methanolic extract was mixed with ferric chloride solution (FeCl2) (1%; w/v),the appearance of greenish-blue color was an evidence for the presence of tannins (Shihata, 1951).

✸Detection of Alkaloids

Aliquot of 0.2 ml of the methanol extract was added to 5ml of 1% HCL using a steam bath, and then 1ml of the filtrate was treated with Mayer's reagent. The appearance of white precipitate was an evidence for the presence of alkaloids (Trease and Evans,1987).

✸Detection of Saponins

Aliquot of 5 ml of the root extract was added to 1-3 ml of mercury chloride solution A white precipitate was developed indicating the presence of saponins (Stahle, 1969).

FTIR Analysis

Nature and chemical structure of the active compounds was examined using the Fourier transformed infrared spectroscopy (FTIR) in order to characterize the chemical nature of compounds. FTIR spectrometry, an advanced type of infrared spectrometry, will give the functional groups that are found in the compound in order to propose a possible chemical structure of the test compound.

Method of Cytotoxicity

Maintenance of the cell lines

Cancer cell lines were monitored to form a confluent monolayer. Sub-culture was established by discarding the old medium. This is followed by washing the cells with sterile PBS under aseptic conditions, then 3 ml of trypsine–versine solution was discarded by washing, using growth medium followed by the addition of new

growth medium, redistributed in special falcons and incubated at 37°C (Freshney, 2000).

Cell Culture and Culture Conditions

HepG2 cell line was used in this study, the cells were grown as a monolayer, spindle like cells. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 mg/ml streptomycin and 1000U/L penicillin. Cell line was grown as a monolayer in humidified atmosphere at 37°C with 5% CO2.The experiments were performed when cells were healthy and at logarithmic phase of growth. HepG2 cell line at passage (40) used in this study they were supplied by Animal cell culture laboratory, Biotechnology Research Center / AL- Nahrain University .

Cytotoxicity Assay

This method was carried out according to Freshney (2000). The cells suspension was prepared by treating 25 cm^3 cell culture flask with 2ml of trypsin solution when a single cell suspension appeared 20 ml of growth medium supplemented with 10% fetal calf serum added to the flask to inactivate the trypsin effect then the viability of the cells counted by using trypan blue dye the viability should be more than 95%. Cell suspension was well mixed followed by transferring 200 µl/well into each well of the 45 well flat bottom micro titer plate using automatic micropipette containing (1×10^5cell/well). Plates were incubated at 37°C until 60 -70% confluence of the internal surface area of the well for Hep

G2 cell line. The cells were exposed to different concentrations of plant extract (800, 400, 200,100, 50, 25, 12.5, 6.2 mg/ml). The negative control wells which contained only the cells with culture media, then the plates were incubated at 37°C in an incubator supplemented with (5%) CO2 for 72 hrs, after elapsing the incubation period, 50 µl/well of neutral red dye were added and incubated again for 2 hrs. The contents of the plate were removed by washing the cells 3 times with PBS then 100 µl elution buffer added to each well (PBS and absolute ethanol 1:1) to remove the excess dye from viable cells. Optical density of each well was read by using ELISA reader at a transmitting wave length on 492 nm, then inhibition rate were determined for each concentration according to the formula (Gao *et al* ., 2003):

$$\textit{Inhibition Rate (\%)} = \left[\frac{\text{Abs.at 492nm of control} - \text{Abs.at492nm of test}}{\text{Abs.at 492nm of control}}\right] \times 100$$

Abs. = Absorance

Results and Discussion

Methanolic Extract

Fifty grams of *Ferula hermonis* powdered roots were used for the preparation of this extract, the PH value for the methanolic extract was 6.2. The weight of the residue obtained after lyophilized was 3.27g, which represents 6.4% of the original roots sample weight. The appearance of the extract was dark brown in color.

Chemical Detection of the Plant Extract

Methanolic extract of *Ferula hermonis* was subjected to chemical analysis to identify the compounds present in extract.

The obtained results indicated the presence of flavonoids , saponins , alkaloids , terpenes , steroids, resins and tannins as shown in Table 1.

Table 1: Chemical detection of some active compounds in *Ferula hermonis* methanolic extract.

Secondary Metabolites	Reagent	Indication	Result of detection
Alkaloids	Mayer ’s reagent	White ppt.	+
Flavonoids	petroleum ether and ammonia solution	Dark green color	+
Tannins	Ferric chloride	Greenish-blue color	+
Resins	Hydrochloric acid	Turbidity	+
Terpenes and Steroids	Chloroform ,acetic anhydride and sulphuric acid	Brown ppt. Blue color	+ +
Saponins	Extract shaking mercury chloride solution	Foam White ppt.	+ +

Note: +ve indicates the presence of the active compound.

Functional groups identification

The FTIR spectrum was used to identify the functional groups of the active components present in plant based on the peaks values in the region of IR radiation. When the plant extract was passed into

the FTIR, the functional groups of the components were separated based on its peaks ratio. The results of FTIR analysis confirmed the presence of carbocylic acid ,alcohol, phenol, alkanes, alkynes, methylene , alkyl halides, aromatic compounds, secondary alcohol, aliphatic amines and tertiary alcohol (figure 1, and table 2).

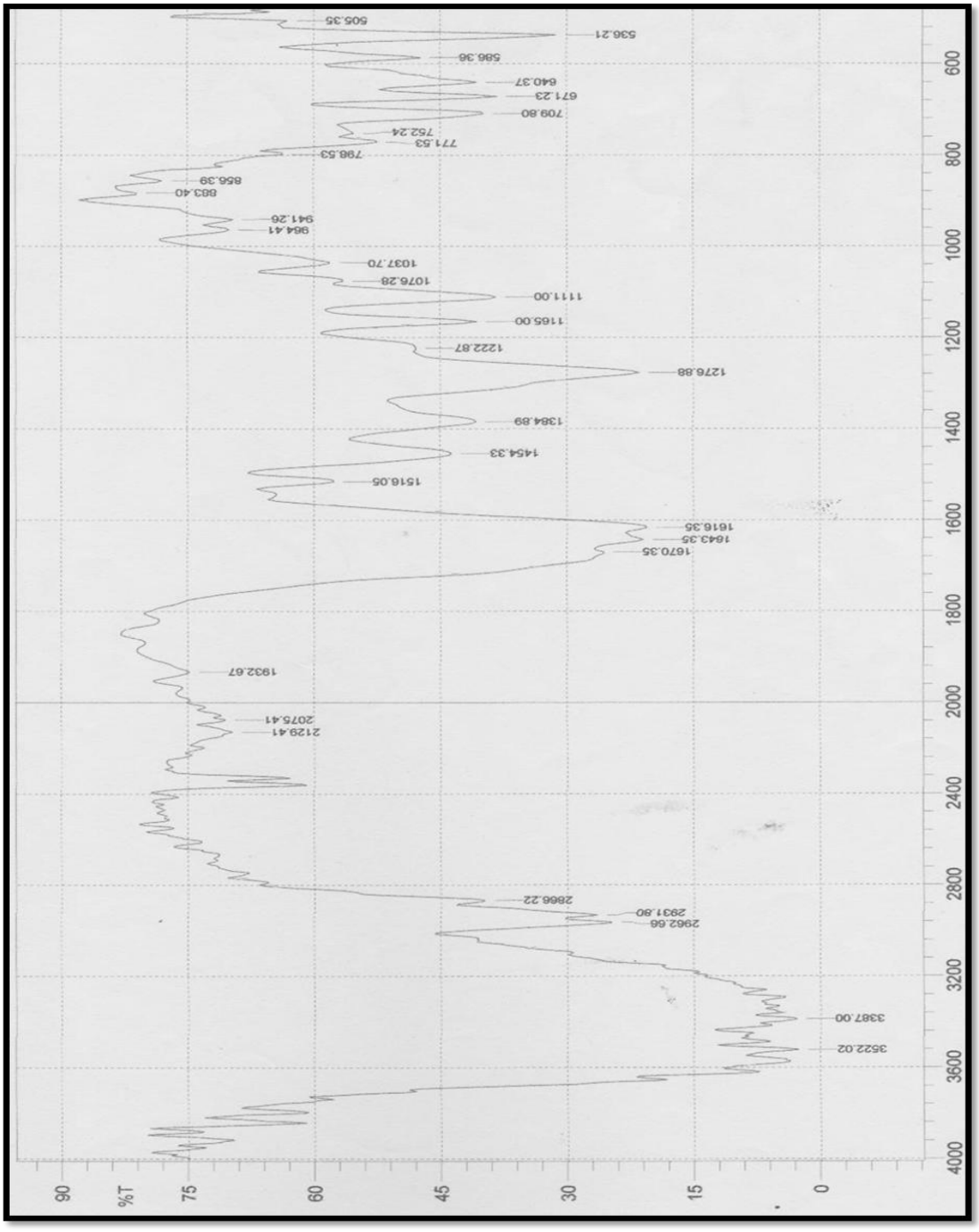

Figure 1 : FTIR Spectrum of *F. hermonis*– Roots extract

Table 2: FTIR Peak Values and Functional groups of *F. hermonis* roots extract.

S. No.	Peaks values	Functional groups
١	.02٣٥٢٢	Carbocylic acid
٢	.00٣٣٨٧	Alcohol, phenol
٣	.66٢٩٦٢	Alkanes
٤	٢٩٣١,٨٠	Methyl
٥	٢٨٦٦,٢٢	Methylene
٦	٢١٢٩,٤١	Alkynes
٧	٢٠٧٥,٤١	Unknown
٨	١٦٤٣,٣٥	Acyclic C=C
٩	١٦١٦,٣٥	Acyclic C=C
١٠	١٥١٦,٠٥	Aromatics C-C
١١	١٤٥٤,٣٣	Aromatics C-C
١٢	١٣٨٤,٨٩	Aromatics C-C
١٣	١٢٧٦,٨٨	Alcohol tertiary
١٤	١٢٢٢,٨٧	Alcohol
١٥	١١٦٥,٠٠	Alkyl halides
١٦	١١١١,٠٠	Aliphatic amines
١٧	١٠٧٦,٢٨	Alcohol
١٨	١٠٣٧,٧٠	Alcohol
١٩	٧٧١,٥٣	Alkyl halides
٢٠	٧٥٢,٢٤	Aromatics C-C
٢١	٧٠٩,٨٠	Alkanes
٢٢	٦٧١,٢٣	Alkanes
٢٣	٦٤٠,٣٧	Alkanes

Spectral differences are the objective reflection of componential differences. By using FTIR spectrum, we can confirm the functional constituent's presence in the given parts and extract, identify the medicinal materials from the adulterate and even evaluate the qualities of medicinal materials (Liu *et al*., 2006).

The results of the present study spectrum also revealed the functional constituents present in the methanolic extract of *F. hermonis*. FTIR exhibited a novel phytochemical marker to identify the medicinally important plant. The presence of alcohols esterified

with aromatic acids are believed the active constituents as the compound ferutinine (Gonzalez and Barrier, 1995).

Cytotoxic effect of Plants Extracts on tumor cell line

The cytotoxic activity of the plant roots extract was determined by evaluating its effect on growth of HepG2 cell line after incubation for 72 hours with different concentrations of extract (800, 400, 200, 100, 50, 25, 12.5, 6.2 mg/ml). Optical density of tumor cell line culture was measured at transmitting wave length of 492 nm. The cytotoxic effect expressed by the percentage of inhibition growth rate (I.R) which represents the cytotoxicity of plant extracts.

Statistical analysis reveals significant decrease of HepG2 cells viability. The effect of *F. hermonis* on cell viability of HepG2 cell line culture show that plant extract cause significant decrease ($p < 0.05$) of HepG2 cell viability, and this decrease in cell viability is increased with the increasing the extract concentrations in comparison with the negative control, and the growth inhibition percentage reach to (78.3%) see figure 2.

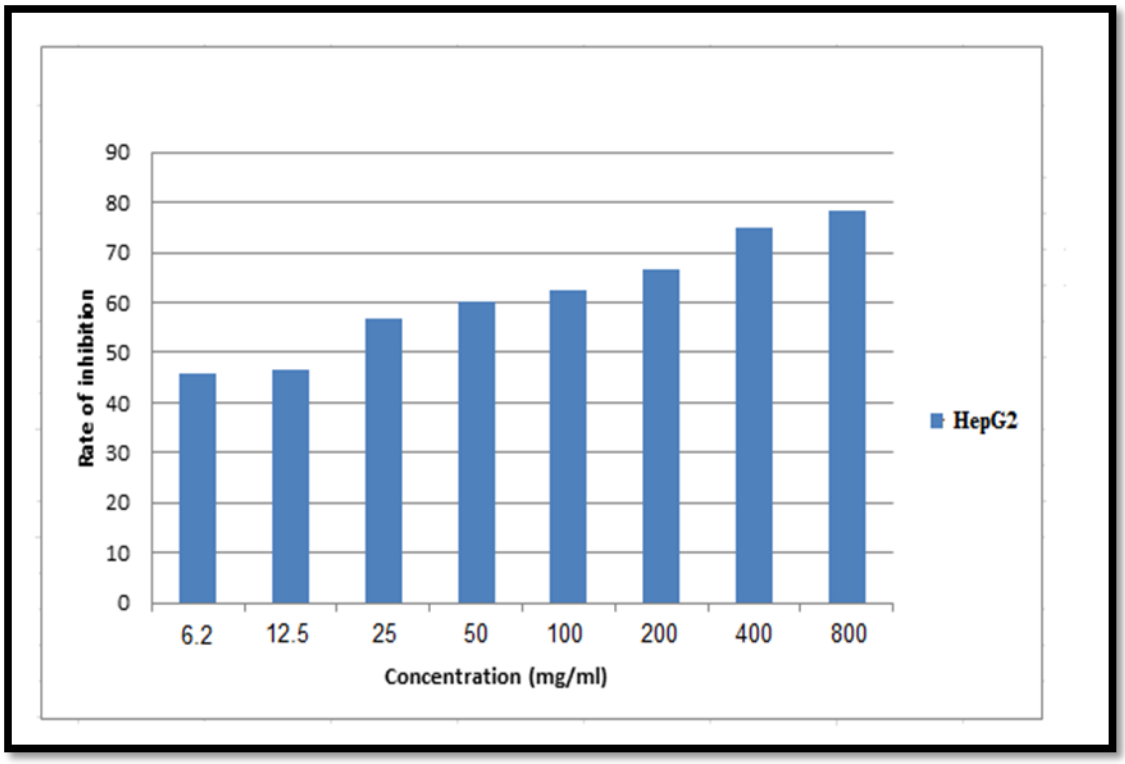

Figure 2: Growth inhibition percentage of *F. hermonis* on HepG2 after 72hr.

Effect of plant roots extract against HepG2 cell line increased with the increase of roots extract concentration until it reaches the maximum effect at the concentration of 800 mg/ml with an inhibition rate of 78.3% at wave length of 492nm and the lowest concentration 6.2 mg/ml exhibited an inhibition rate of 45.8% at wave length of 492nm.

Zychlinsky, (1999) indicated that tumor cells differ in morphology than normal cells and one most important difference is that tumor cells highly express receptors on their membranes than normal cells which may allow the attachment of different components. In addition to that, DNA of tumor cell found in relaxant shape, and the DNA molecule was found in unstable figure because of the far away between the H-bond which connect the both strands of DNA. This makes it easy for compounds to interfere or to be associated with both strands of DNA Whereas DNA of normal cell has a strong H-bond connect the both strands to each other and make it more stable, so the compounds cannot interfere or associated with DNA strand (Belijanski, 2002).

Cell cycle progression is an important biological events having controlled regulation in normal cells, which almost universally becomes aberrant or deregulated transformed and neoplastic cells. In this regard, targeting deregulated cell cycle progression and its modulation by various natural and synthetic agents are gaining wide spread attention in recent years to control the unchecked growth and proliferation in cancer cells. It was show that many phytochemicals halt are uncontrolled cell cycle progression in cancer cells. Among these phytochemicals, natural sesquiterpenes have been identified in *F. hermonis* as a one of the major classes of natural anticancer agents through cell cycle blockade in G1 and induction of dose-dependent apoptosis (Barthomeuf *et al*., 2008).

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