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Cytotoxic Activities Of Ferulago Abbreviata Total Flavonoid Extract On Three Different Cancer Cell Lines

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Abstract

The ability of various medicinal plants to generate natural chemicals varies. The total flavonoid compounds and cytotoxic activity of aerial parts of Ferulago abbreviata harvested from Piramagrun Mountain in Iraq's northeast Kurdistan region were investigated for the first time. Total flavonoid compounds (TFCs) were extracted using 10 percent hydrochloric acid. The aluminum chloride technique and high-performance liquid chromatography were used to analyze the plant's total flavonoids extract. This research compared the cytotoxic effects of total flavonoids from F. abbreviata aerial parts on normal epithelial cells (HBL100) against three lines of cancerous human cells: colorectal carcinoma (HCT 116), lung adenocarcinoma (A549), and cervical carcinoma (HeLa). MTT assays were performed to evaluate the cytotoxicity and cell viability of the TFC. HPLC revealed several peaks with varying retention periods; isorhamnetin had the greatest concentration (26.22 µg/mg of extract), while chlorogenic acid had the lowest (0.08 µg/mg of extract). The findings of this research suggest that the cytotoxic effects of F. abbreviata TFC products against A549, HCT116, and HeLa cell lines are linked to apoptosis induction. TFCs, on the other hand, had no discernible impact on the normal epithelial cell line (HBL100). Because of its cytotoxicity, it may be a promising candidate for the creation of chemopreventive drugs that induce death in cancer cells. The current research is the first to look at the cytotoxicity of F. abbreviate aerial parts TFC extracts against the A549, HCT116, and HeLa cell lines.

Keywords: Lung adenocarcinoma (A549), Colorectal carcinoma (HCT 116), Cervical carcinoma (HeLa), Anticancer agent, MTT tests

INTRODUCTION

Due to their organic characteristics, particularly secondary metabolites, medicinal plants have been utilized in chemotherapy. Numerous research have been conducted recently with the goal of discovering and isolating novel medicinal chemicals from plants (Chelalba et al. 2021).

Herbal medicines have been utilized for a long time and are still used as the main medical therapy in poor nations. Plants' natural antibacterial properties have long been used in medicine (Hussein et al. 2021). As a result, researchers are now examining the characteristics and applications of plant extracts in the development of new nanomaterial-based treatments for a variety of

illnesses, including cancer (Jaafar, Al-Saffar, and Yousif 2020). Numerous plant species are already being utilized in the treatment and prevention of malignant cell development in the body (Salih, Alobaidi, and Alobaidi 2015). An extensive study of plants used in traditional medicine in poor nations turned up many species that have anticancer effects(Greenwell and Rahman 2015).

Herbal medicines are increasingly being used in cancer treatment because they are more medically friendly, better aligned with their target areas, and less damaging to healthy cells. Furthermore, anticancer medicines produced from natural products have been shown to offer different methods of inducing cell death (Shamsee et al. 2019). This is important to know. Many scientists are now focusing their studies on the plants' ability to provide natural products to the pharmaceutical sector in light of these facts (Seca and Pinto 2018).

In every nation, cancer is the top cause of mortality and a major impediment to improving life expectancy (Bray et al., 2021). According to World Health Organization (WHO) estimates, cancer would be the leading cause of death for persons under the age of 70 in 112 countries in 2019 and the third or fourth leading cause of death in another 23 countries (Sung et al. 2021). Some cancer therapies, such as surgery, radiation, and chemotherapy, are unsuccessful now. One of medicine's and pharmacology's primary goals is to find novel, effective treatments and anticancer medicines (Dorai and Aggarwal 2004; Sarvmeili, Jafarian-Dehkordi, and Zolfaghari 2016; McWhirter, Oakes, and Steyn 1996).

Ferulago angulata (Schlecht) Boiss can be found in northern Iraq, Iran, and eastern Turkey. It is divided into two subspecies: F. angulata (Schlecht) and F. carduchorum (Boiss and Hausskn) or F. abbreviata (Süzgeç-Selçuk and Dikpınar 2021). F. abbreviata has yellow blooms with thin leaves and grows to a height of 50–160 cm. Ferulago species are utilized in traditional medicine for their digestive, tonic, sedative, and aphrodisiac qualities, and have been used to cure intestinal worms and hemorrhoids in Turkey since ancient times hemorrhoids (Sadeghi et al. 2016). Boissier classified the Ferulago species for the first time (Akalin Uruşak and Kizilarslan 2013).

There are several phytochemicals found in plants, each of which may have a variety of diverse biological impacts on the human body. Different routes are used by these phytochemicals, making them potential anticancer metabolites (MIRZA, Akrami, and Mansouri 2014). There have been tens of thousands of plant species investigated in the last several years to find new cancer-fighting agents (Heidari et al. 2014). Coumarins, a class of phytochemicals, are drawing attention because of the wide range of biological actions they exhibit. For high plants, the Apiaceae family is among the most significant coumarin-producing groups with more than 13,000 coumarins discovered to date; it is also one of the most common coumarin-producing plants (Venugopala, Rashmi, and Odhav 2013). Antioxidant, anti-inflammatory, anticancer, monoamine oxidase B inhibitory, antimicrobial, anti-allergic, hepatoprotective, antithrombotic, and antiviral activities are just some of the natural coumarins' many properties (Kostova 2005; Salem et al. 2013; Gaudino et al. 2016; Kozioł and Skalicka-Woźniak 2016; Thomas et al. 2017). Because of the functional groups in the original structure, they also exhibit strong anticancer capabilities with little side effects. Several cellular pathways may be affected (Sandhu et al. 2014), and researchers from all around the globe are working hard to figure out how to induce apoptosis in malignant cells (Heidari et al. 2014).

Ferulago W. Koch species showed anticancer efficacy when tested against a variety of tumor cell lines. Some coumarin derivatives identified and tested also yielded encouraging results in these investigations, which were conducted on extracts of plant species made using various solvents. It has been extensively studied for its ability to affect cancer cell lines. Anticancer effects were revealed in research by Amirghofran et al. (Amirghofran et al. 2006) on K562 leukemia cell lines and the growth

of Jurkat cells by extracts made from aerial plant parts. Extraction of F. angulata species was shown to inhibit the proliferation of three leukemia and lymphoma tumor cell lines in a dosage and time-dependent manner, whereas peripheral mononuclear cells were not substantially impacted during this procedure (Shahneh et al. 2013).

In the light of these studies mentioned above performed with various extracts obtained from different Ferulago species, we decided to investigate the cytotoxic activity of the flavonoid's compounds extracted from the aerial part of Ferulago abbreviata growing naturally in Iraq against some different cancer cell lines (lung, colorectal, and cervix carcinoma using A549, HCT 116, and HeLa cell lines) as a preliminary study.

MATERIALS AND METHODS

Plant material

The aerial parts of Ferulago abbreviata were collected in June-July 2019 from Piramagrun (spelling variants: Piramagroon, Birah Magrun) mountain, northeast Kurdistan region (Iraq). According to the morphological description, the plant was botanically identified as F. abbreviata by Dr. Saman A. Ahmad, field crops Dept., college of Agriculture Science, Sulaimani University. Furthermore, the F. abbreviata CC (Towns) specimen was identified and deposited in the medical plant's research center herbarium of the Kurdistan Botanical Foundation (KBF) in Sulaimani, Iraq and submitted to NCBI GenBank under accession number MZ521030.

This plant grows and develops between June and August at the mountain's top, a rocky place; cliffside eroded sandy soil. Its stems are 1-1.5 m tall with strong sulcate leaves 2-pinnately compound all, but final division very elongated, canaliculated with club-shaped hairs. After being air-dried in the shade at room temperature, the aerial plant parts were used for flavonoid compounds extraction.

Extraction of Total Flavonoids from F. abbreviata

A crusher was used to grind 50 grams of dried aerial parts into a fine powder. Following that, 250ml of 10% hydrochloric acid (HCl) was added to the plant powder in the flask and well mixed. The mixture was then transferred to the reflux device and heated to 45°C for 15 hours. After that, the extract was filtered using filter paper. The aglycone moiety was extracted three times with 30ml ethyl acetate using a Separatory Funnel. The collected layers of ethyl acetate were rinsed with 75ml of distilled water to eliminate redundant acids. It was then put into a petri dish and dried by incubating it at 40ºC in the oven. The extracted flavonoids reflected the total flavonoids.

Detection of Total Flavonoids

The total flavonoid content was determined using a colorimetric aluminum chloride assay (Al-Osaj, Al-Sammarraei, and Al-Osaj 2016). Rutin was used as a standard (0.5, 0.25, and 0.12 mg/ml in 50% ethanol). An aliquot of 1ml from each concentration of standard rutin solution and the extracted flavonoids (10mg/ml) was placed into glass tubes, followed by 0.75 ml of 5% sodium nitrite solution and thorough mixing. 1.5 ml of 10% $AlCl_3$ in 50% diluted ethanol was added to each tube, shaken well, and allowed to remain at room temperature for an additional 5 minutes. Finally, each tube was filled with 5ml of 1N NaOH solution. A spectrophotometer set to 510nm was used to determine the absorbance. A standard curve was constructed between each concentration and absorbance; the total flavonoid quantity as rutin was then estimated using the straight-line equation

derived from the plotted curve. The total flavonoids in the extract were reported as mg of rutin equivalents per gram of dry weight.

High-performance liquid chromatography (HPLC) of Total Flavonoids

To prepare the total flavonoids for HPLC, 1 mg/ml was dissolved in 70% methanol and filtered through a Millipore 0.22-micron filter. The F. abbreviata sample was tested using high-performance liquid chromatography (HPLC) to determine the active component. Shimadzu's LC-2010A HT Liquid Chromatograph is the HPLC model. The chromatographic test was carried out on a C18 column (250mm x 4.6mm x 0.5Mm), and elution was carried out in a gradient system using ACN:0.5 % Formic acid (70:30) (solvent A) and ACN:0.5 % Formic acid (30:70) (solvent B) at a flow rate of 0.5 mL/min. At 340nm wavelengths, the peaks were observed. The injection volume was 20µl, and the temperature was always maintained at 25 °C. To detect flavonoids, retention periods and analysis of extracted flavonoids curves with standards solutions were employed. This equation was used to quantify flavonoids:

The concentration of sample = Area of sample/ Area of standard x concentration of standard

Determination of Cytotoxicity

The cytotoxic effects of total flavonoids derived from F. abbreviata were investigated using the MTT assay. The total flavonoids' effect was studied on the cell viability of three cancer cell lines (Pulmonary adenocarcinoma A549, Colorectal carcinoma HCT 116, and Cervix carcinoma HeLa) in comparison to normal cell lines (HBL 100).

Maintenance of cell culture:

RPMI-1640 media supplemented with 100 units/mL penicillin, 100g/mL streptomycin, and 10% fetal bovine serum was used to maintain cancer cell lines. Cells were passaged twice weekly with Trypsin EDTA, reseeded at 80% confluence, and incubated at 37°C.

Measurement of the Viable cell by MTT Assay(Mohammed, Alsamarrae, and Hamza 2015)

A colorimetric assay using 3- [4, 5]-dimethylthiazoyl]-2, 5-diphenyltetrazolium bromide (M.T.T. dye) was used to determine cell viability according to the manufacturing company (Elab science MTT kit, USA). In each of the 96 well microtiter plates (10^4 cells/well), aliquots of 100µl suspended cells were grown. For 24 hours at 37ºC in a CO2 incubator, the microtiter plate was incubated. Serial concentrations of plant extract (TFC) were produced from each 400µg/ml stock solution to get 200,100, 50, 25, 12.5, and 6.25µg/ml, which were subsequently sterilized using a 0.22µm Millipore filter. Following that, 100µl of each extract concentration was applied to each well of the cell line seeding plate in triplicate. 24 hours at 37°C were spent incubating the microtiter plate in a CO2 incubator. Following 24 hours, 50µl of M.T.T. dye (1X) was added to each well and incubated for an additional four hours. A fine gauge needle was used to remove the medium carefully. Because the MTT-formazan crystals are produced only by living cells, 150µl of D.M.S.O. was added to each well and incubated at 37ºC for 15 minutes with shaking to dissolve them. An ELISA reader was used to instantly measure the absorbance at 570nm. The mean absorbance for each group of triplicates was calculated.

The following equation was used to calculate the rate of cell growth inhibition (percentage of cytotoxicity):

Inhibition rate = A- B/A x 100

where A is the optical density of the control, and B is the optical density of the samples (Albukhaty et al., 2018).

Cell shape visualization

Under an inverted microscope, the crystal violet dye was utilized to observe the cell shape. Cells were seeded at a density of 105 cells/mL onto 24-well microtitration plates and incubated at 37°C for 24 hours. After 24 hours, cells were treated with TFC extract at their IC50 concentrations. Following the exposure period, the plates were dyed with crystal violet. The dye was then carefully rinsed off with tap water until it was completely gone. The cells were examined at a magnification of 100X using an inverted microscope and then photographed with a digital camera attached to the microscope (Waheeb, Sulaiman, and Jabir 2020; Al-Shammari et al. 2020).

Statistical analysis:

The collected data were statistically evaluated using GraphPad Prism 6 and an unpaired t-test. The mean and standard error of triplicate measurements were used to calculate the results (SEM). Within the same analytical program, the IC50 values were determined using linear regression analysis.

Results and Discussions

The spectrophotometric absorbance of total flavonoids extracts and rutin standard solutions was recorded at 510nm, as shown in Table 1. Results indicated that the total flavonoids were 7 mg in 1gram of extracted flavonoids according to the straight-line equation (Figure).

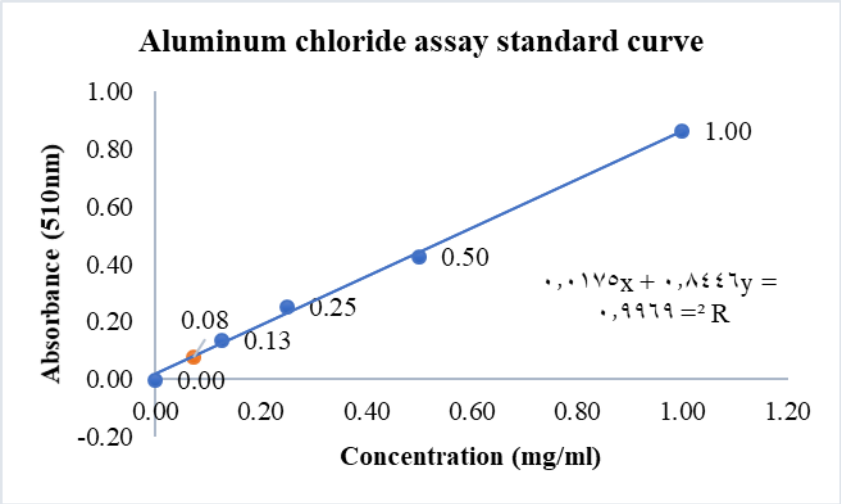


Figure 1. Standard curve for Rutin as determined spectrophotometrically at 510 nm.

Table 1. Absorbance and concentration results of aluminum chloride assay.

ID	Type	Conc [mg/ml]	Abs	Abs- Blank
Stock	Standard	1.00	0.92	0.86
R1	Standard	0.50	0.49	0.43
R2	Standard	0.25	0.31	0.25

R3	Standard	0.13	0.20	0.14
Blank	Standard	0.00	0.06	0.00
Calculated TFC	Unknown	0.07	0.14	0.08

(Ebadi, Mollaei, and Khurizadeh 2019) used Ultrasonic bath at 40 kHz to extract phenolic acids from various F. angulata dry powder portions using a 70% methanol-water solution. They discovered that the unripe seed had the greatest amount of total flavonoids (18.46 mg/g of extract), while the leaves contained the lowest amount (5.49 mg/g of extract); their finding was the closest to ours.

Ather study by (Kiziltas et al. 2017) measured the total flavonoid contents in the flowers of F. angulata (Schlecht.) Boiss. The total flavonoid content evaluated in methanol extracts was 37.4 ± 2.9 mg/g used quercetin as a standard; these results differed from ours.

High-performance liquid chromatography (HPLC)

According to the HPLC analysis of Total flavonoids, compounds of F. abbreviata, the results indicated the presence of these 12 compounds numerate in (Figure), Isorhamnetin had the highest concentration (26.22 µg/mg), while Chlorogenic acid had the lowest concentration (0.08 µg/mg).

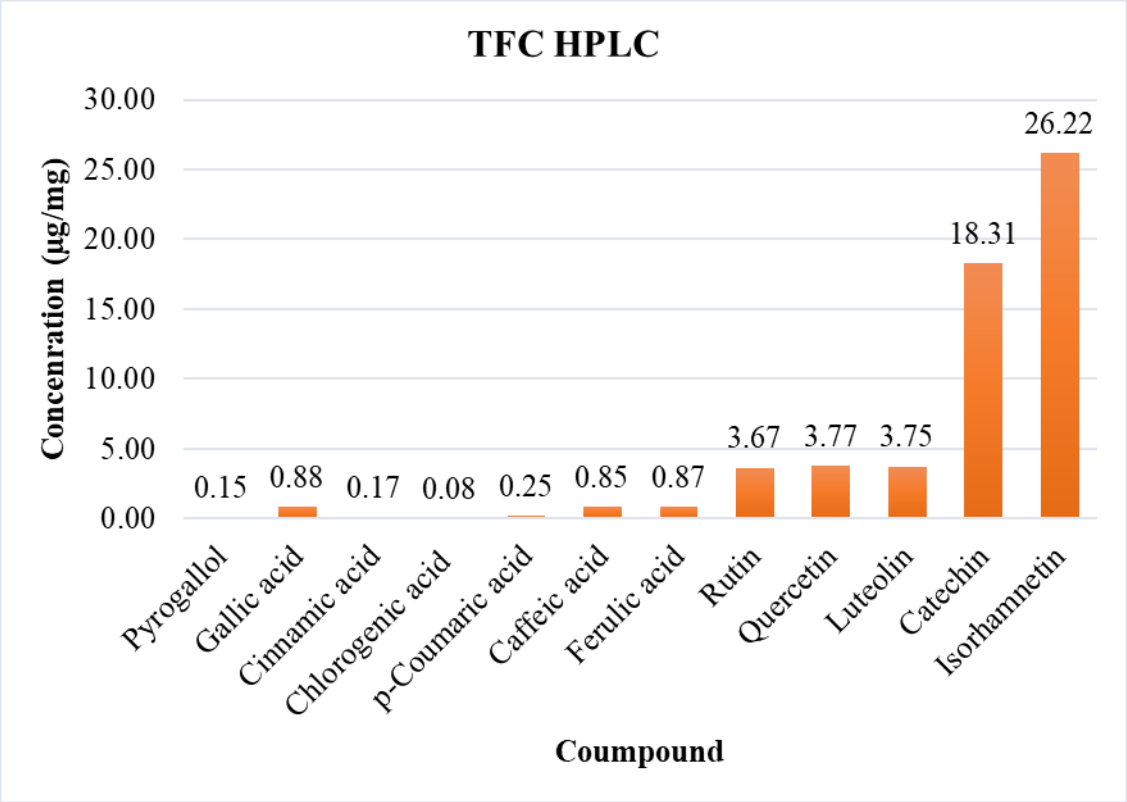
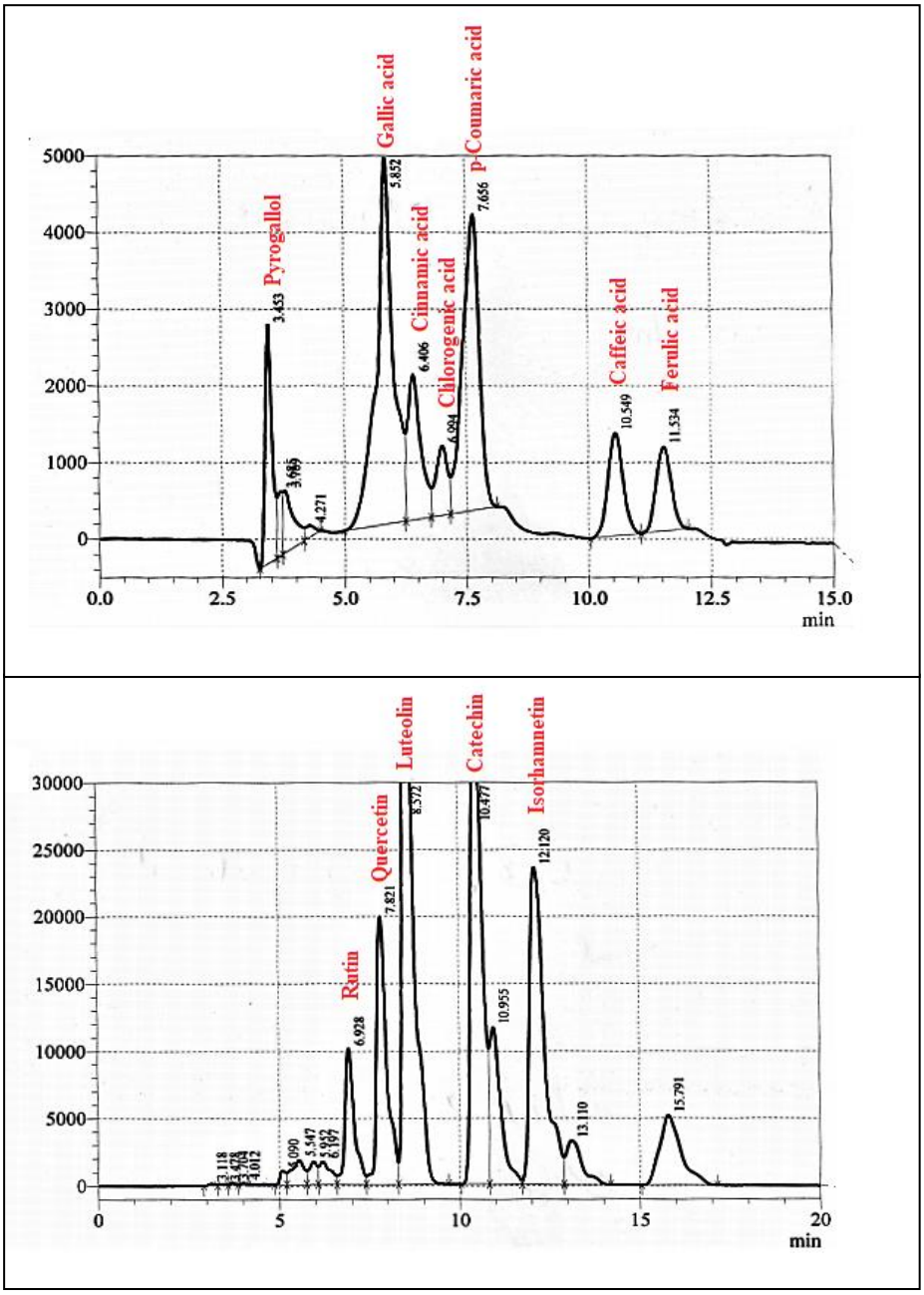


Figure 1. HPLC results of TFC of F. abbreviata.

Compared with the typical chromatogram of Flavonoid compounds standard, the results showed different peaks with different retention times for the total Flavonoid (TF) sample (Figure). Isorhamnetin compound was the highest concentration (26.21µg/mg), while Chlorogenic acid compound was the lowest one (0.08 µg/mg). Isorhamnetin has many different pharmacological effects. Pharmacological effects: Anti-osteo porosity, immune regulation, and other things like that.

Mechanisms of action are mainly anti-inflammatory, antioxidative, and apoptosis-controlling, but they also have other effects. These pharmacological effects can be essential in the treatment of different diseases, like the antioxidative and anti-inflammatory effects of isorhamnetin on acute kidney injury and acute fulminant hepatitis in mice, which are both caused by oxidative stress. Isorhamnetin, on the other hand, can stop HIF-1 from causing cancer cells to move and invade in vitro. Many of isorhamnetin's many effects play a big part in how PI3K/AKT and other signaling pathways and cytokines work to treat diseases. This shows that it has a lot of medicinal value (Gong et al. 2020). Apoptosis (cell death) is induced in gastric cancer cells by isorhamnetin, which has antitumor effects as well (Li et al. 2021). Scientists hypothesize that the anti-inflammatory properties of isorhamnetin in asthma are due to its ability to modulate the MAPK and NF-B signaling mechanisms, which inhibits TNF-a induced inflammation, proliferation, and migration (Ren et al. 2021). It was discovered that isorhamnetin had an antiproliferative effect on oral squamous cell carcinoma (OSCC), and that it could cause cell cycle arrest in the G2/M phase and promote cytoplasmic vacuoles, which were attributed to paraptosis cell death, which was mediated by reactive oxygen species (ROS) and the Extracellular Signal-Regulated Kinases (ERK) pathway (Chen et al. 2021).

In a study by Zhai and his team, they found that isorhamnetin has both biological and clinical value in the development of gallbladder cancer (GBC). They show that isorhamnetin can stop the



growth and spread of GBC cells without having any negative effects. They did this by downregulating the PI3K/AKT signaling cascade(Zhai et al. 2021).

Figure 2,3 HPLC peak results of TFC sample

Effect of F. abbreviata total flavonoids extracts on Cell viability

The M.T.T. assay was used to evaluate the cytotoxicity of F. abbreviata TFC products on three cancer cell lines (Pulmonary adenocarcinoma A549, Colorectal carcinoma HCT 116, and Cervix carcinoma HeLa) in comparison to a normal cell line (Epithelial cell HBL 100). As shown in Figure , cancer cells treated with F. abbreviata extract had a substantial decrease in cell viability when compared to control cells. Additionally, F. abbreviata TFC extract at concentrations ranging from

6.25 to 200µg/ml had a strong dose-dependent inhibitory impact on A549, HCT116, and HeLa proliferation, suppressing proliferation by more than 75%. The extract, on the other hand, had no discernible effect on the growth of normal HBL100 cells.

The study's findings indicated that TFC extracts were cytotoxic to all cells except the control (HBL100) at a concentration-dependent rate. In summary, the TFC extract demonstrated significantly more cytotoxic activity with a P-value of 0.0008 against cancer cells.

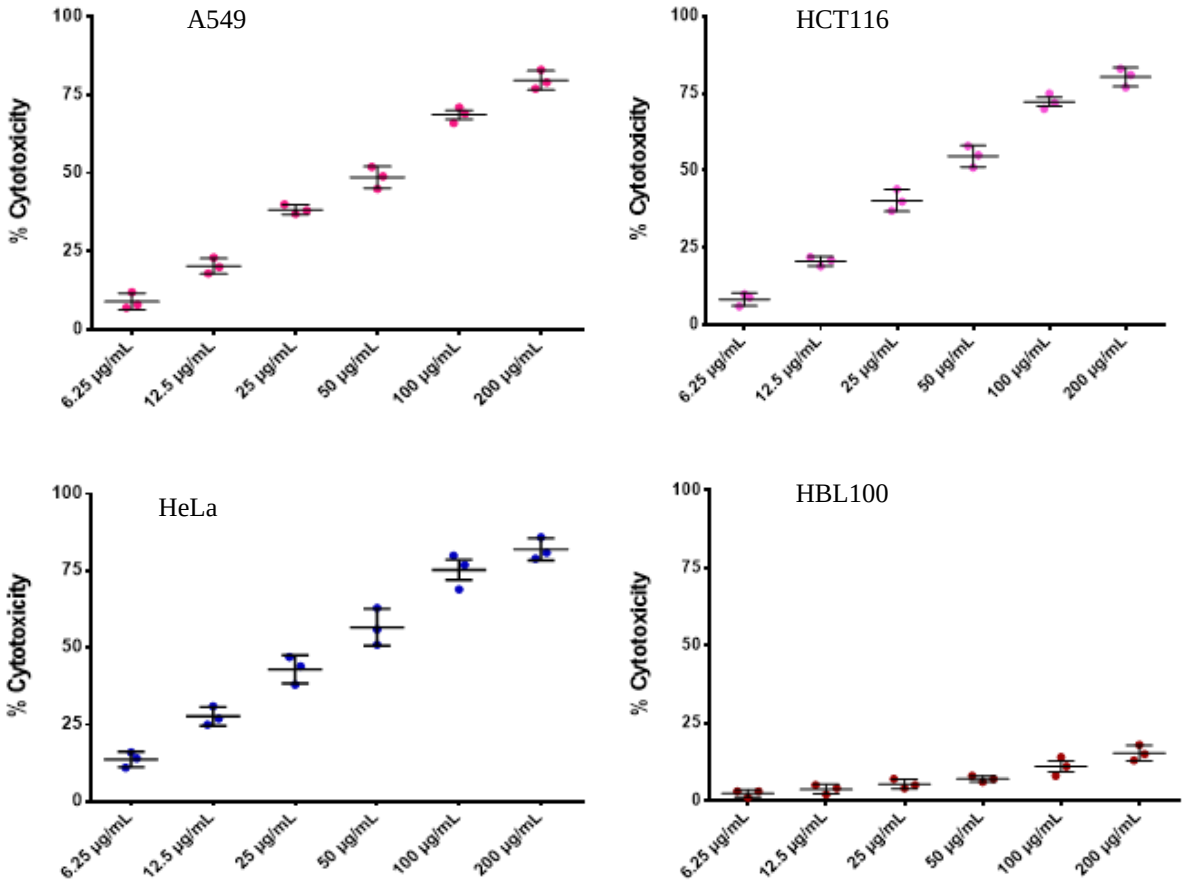


Figure 4. Effect of different concentrations (6.25-200µg/ml) of F. abbreviata TFC extracts on Four cell lines. Values represent the mean of three experiments.

The doses of F. abbreviata TFC that inhibit growth by 50% (IC50) against the three cancer cell lines after 24 hours are presented in Figure . Proliferation of the HeLa cell line was most effectively inhibited, with an IC50 value of 27.37µg/ml. In comparison, the HCT 116 cell line was substantially decreased, with IC50 values of 35.78µg/ml.

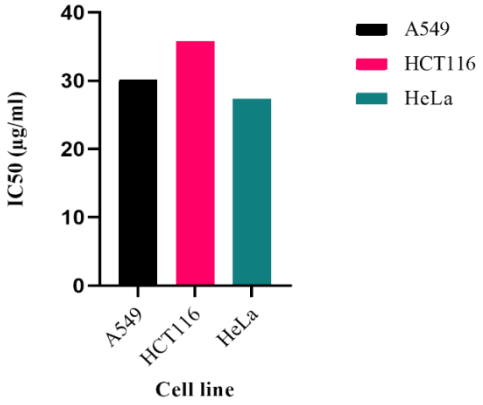


Figure 5. IC_{50} concentration of TFC against three different cancer cell lines after 24 hours of treatment. Abbreviations: IC50, half-maximal inhibitory concentration.

The cells' shape under an inverted microscope after treatment with IC_{50} of TFC extracts using crystal violet stain in contrast control cells (Untreated cells) is a preview in Figure .

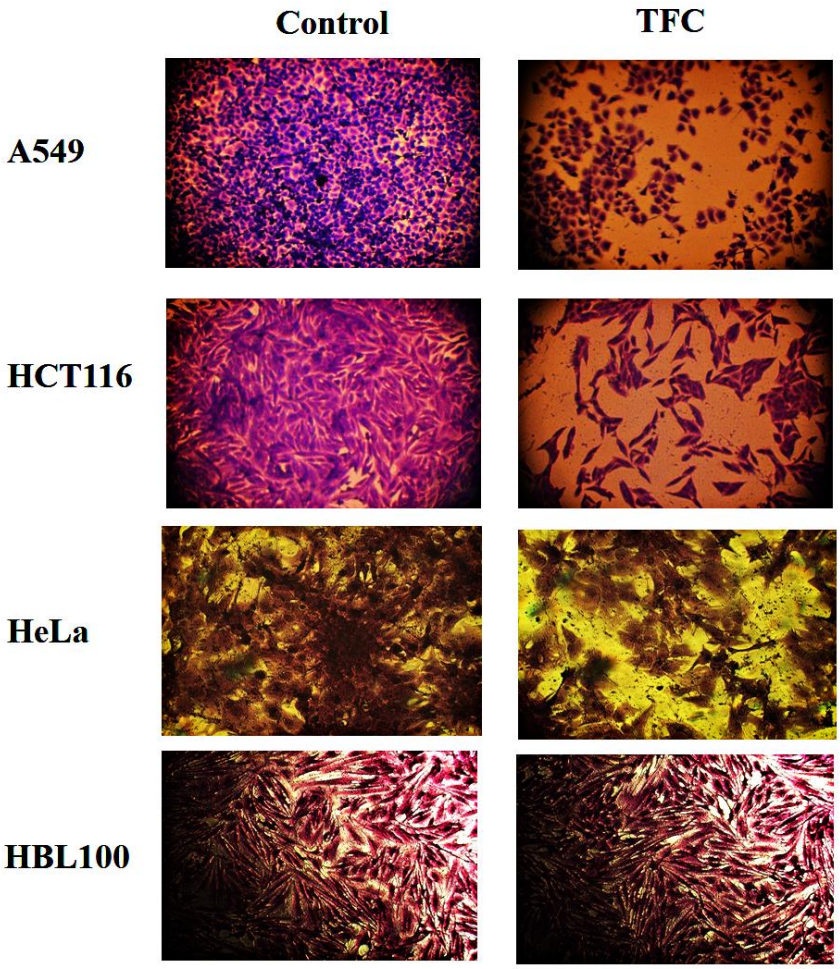


Figure 6. Cell shape visualization under an inverted microscope (10X and 40X). TFC: total flavonoids compound.

According to many scientific research, Ferulago species display a wide range of behaviors. Currently, the literature on all species' anticancer activities is insufficient. The Iraqi flora estimates that the Ferulago genus has around 50 species worldwide, including five taxa unique to Iraq. The current study examined the ability of F. abbreviata extracts to attack several kinds of cancer cells. Some research on the anticancer properties of Ferulago species has also been published in the literature (Dall'Acqua et al. 2011; B.A 2014; Dall'Acqua et al. 2014; Golfakhrabadi et al. 2015; Lorigooini et al. 2019).

This is the first research to investigate the effects of a flavonoid extract from F. abbreviata on cell lines such as A549, HCT116, HeLa, and HBL. Using the MTT test, this study discovered that F. abbreviata TFC extracts substantially reduced the growth of cancer cell lines in vitro. Additionally, this antiproliferative action of F. abbreviata extract was dose-dependent and tumor-selective, as shown by the relatively low IC50 values and lack of substantial effects on normal HBL100 cells, respectively (Figure). Our results were like Fatemeh, Zare et al., and Heidari et al., finding that the TFC had no cytotoxic effect on normal cell lines, which might be due to increased nucleosomes, which inhibit cancer cell line growth via an apoptosis-dependent pathway (Shahneh et al. 2013; Akrami et al. 2014).

In their studies on Ferulago species, Bakarates et al., Karimian et al., and Rezaei Dezaki et al. found that TFCs had cytotoxic effects on cancer cell lines because they inhibited the cell cycle and increased Annexin V binding, leading to apoptosis via a mitochondrial-dependent pathway or an apoptosis-dependent pathway (Rezaei Dezaki et al. 2019; Karimian et al. 2015; Bakar-Ates et al. 2021). According to Mirzaghaei et al., another cytotoxic impact may be via the inhibition of sprouting, tube formation, and migration, resulting in anti-angiogenicity (MIRZA, Akrami, and Mansouri 2014). As a consequence, the leaves contain antiangiogenic chemicals, making it a potential candidate for anti-angiogenesis treatments in tumors.

The compounds xanthotoxin, isoimperatorin, and oxypeucedanin, among others, have been found in Ferulago species and have been shown to be highly cytotoxic to a variety of cell lines, according to Ameen (B.A 2014).

Conclusion

According to our results, total flavonoids derived from aerial parts of F. abbreviata have a highly cytotoxic effect on cancer cells and a slight cytotoxic effect on normal cells in the laboratory. To the best of our knowledge, this is the first study to demonstrate that TFC extracts from aerial portions of F. abbreviata are cytotoxic to the cancer cell lines HCT 116, A549, and HeLa. Because it is toxic to malignant cells rather than normal cells, F. abbreviata extract may promise an anticancer agent. This is one of the most challenging difficulties in creating anticancer medications because it is harmful to malignant cells rather than normal cells.

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