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The essential oils isolated from the fruit of ficus carica induced reactive oxygen species and apoptosis of human liver cancer cells

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Abstract

Ficus carica is an edible fruit that has numerous health benefits for humans. The essential oils from ficus carica fruit (FCF) were isolated using a hot distillation procedure with water (FCFW) and hexane (FCFH) solvents, and the chemical contents were characterized using gas chromatography-mass spectrometry. The MTT assay and flow cytometry were used to investigate the anticancer activity against human liver cancer (HepG2). According to gas chromatography analysis and the NIST library, the chemical compositions of FCFW were main seven recognized compounds belong to chemical class of nitrogenous, aldehyde, acid, and ketone compounds, while FCFH was main two known compounds, belongs to chemical class of aldehyde, and nitrogenous compounds. The cytotoxicity test showed that FCFW and FCFH have similar IC_{50} values (40 %v/v). The IC_{50} value was utilized to detect cell cycle arrest in S phase for FCFW, reduced reactive oxygen species, and late apoptosis. These findings demonstrated that FCFW essential oils were more effective than FCFH essential oils. Merit investigations may lead to an increase in the medicinal relevance of FCFW essential oils.

Keywords: *ficus carica* fruit, essential oils isolation, flow cytometry, human liver cancer, MTT assay.

Introduction

Proteins, lipids, polysaccharides, essential oils, phenols, flavonoids, and alkaloids are all good sources of healthful natural big and small molecules that could be used for medical purposes**[1,2]**. Among these natural chemicals are essential oils, which have been found to have beneficial effects on human health**[3,4]**. Essential oils are plant components isolated through distillation (through steam and/or water) or mechanical processes such as cold pressing. Aromatherapy, a type of alternative medicine that uses plant extracts to enhance health and well-being, frequently includes essential oils. However, some of the health claims made about these oils are debatable**[5]**. Around 3000 different essential oils have been represented, with around 300 being used economically in the seasoning and smells market**[6]**. Popular oils include peppermint (used to promote energy and aid digestion), lavender (used to relieve stress), and sandalwood (used to relieve pain) (used to calm nerves and help with focus). Essential oils have a variety of biological effects, including antibacterial, antifungal, and anticancer activities**[7-11].** Due to the absence of target specific release, research on essential oils as anticancer therapeutic agents is still in its early stages, and the great potential of essential oils needs to be exploited. To make the treatment more effective, investigations including clinical trials are required, as well as the application of improved technology for the targeted organ-specific release of essential oils**[9,10]**. Distillation, sometimes with the use of steam, is the most common method for isolating essential oils or volatile oils. Expression, solvent extraction, absolute oil extraction, resin tapping, wax embedding, and cold pressing are some of the other techniques. They are used in fragrances, cosmetics, soaps, air fresheners, and other items, as well as to flavor food and beverages and to scent incense and home cleaning products**[11,12]**. Essential oils should not be mistaken with perfume, scent, and other similar products because the latter typically contain pure chemical components, whereas essential oils are derived from plants. Aromatherapy is a sort of complementary medicine in which aromatic compounds with therapeutic qualities are used. Aromatherapy can help you relax, but there isn't enough evidence that essential oils can effectively treat any condition. Poor essential oil use can cause allergic responses, inflammation, and skin irritation,

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and children are especially vulnerable to the hazardous effects of improper use**[13]**.

The *ficus carica* is a delectable fruit with numerous health benefits for humans, including digestive, endocrine, reproductive, and respiratory system illnesses, laxative, cardiovascular, respiratory, antispasmodic, and anti-inflammatory medicines. It is also used to treat gastrointestinal and urinary tract infections**[14,15]**. Phytochemical analysis of *F. carica* leaves, roots, fresh and dried skin, pulps, and peels reveals a wide range of bioactive compounds including phenolic, phytosterols, organic acids, anthocyanin, triterpenoids, coumarins, and volatile compounds (hydrocarbons and aliphatic alcohols), as well as a few other classes of secondary metabolites. As a result, the majority of *F. carica* species contain phenolic compounds, organic acids, and volatile chemicals**[16,17]**. Because it is fat and cholesterol free and has a large quantity of amino acids, it is also a wonderful source of minerals, vitamins, carbs, and dietary fibre**[18]**. The majority of pharmacological studies on *Ficus carica* were conducted with uncharacterized crude extracts; it exhibited various biological activities such as antioxidant, anticancer, antibacterial, antifungal, hepatoprotective, hypoglycamic, hypolipidemic, antipyretic, antituberculosis, irritant potential, nematicidal, antispasmodic, antiplatelet, anthelmintic, analgesic, and analgesic**[19]**. Essential oils from *ficus carica* seeds have been shown to have health benefits such as protecting the eyes, preventing macular deterioration, lowering the risk of cardiovascular disease, promoting the proper functioning of the liver and kidney, preventing oxidation and cell damage, antidiabetic properties, and significantly improving skin quality**[20,21]**. To the best of our knowledge, no studies have been conducted to investigate the anticancer potential of *ficus carica* essential oils.

As a result, in this study, employed water and hexane solvents to isolate essential oils from *ficus carica* fruit in order to identify the chemical compositions and explore anticancer properties against human liver cancer (HepG2) using the MTT assay, cell cycle, reactive oxygen species, and apoptosis. The findings will boost the medicinal value and use of essential oils derived from the *ficus carica* fruit.

Experimental

Ficus carica fruit collection

Four kilograms of *ficus carica* were obtained from a hit market during the crop season, which runs from August to October, 2020. This *ficus carica* fruit is grown in Karbala Governate, Iraq, where the soil is not saline and suitable environmental for growing a variety of fruits. It's authenticated and deposited in the Faculty of Agriculture, University of Basrah, Iraq with the voucher specimen number of 250-4. The fruit is cleaned, cut into small pieces, dried for two weeks, milled, and stored in covered opaque glass containers in the refrigerator at 4 °C until needed.

Essential oils isolation method of *ficus carica* fruit

Using water and hexane solvents, we isolated essential oils from ficus carica fruit using the method reported in references **[22,23],** with some modifications:

Isolation using water: 50 g of *ficus carica* fruit powder was added in the round flask, followed by 500 mL of water, and distilled in a Clevenger apparatus for 3 h. The isolation processed during three hours of distillation. The essential oils were isolated using separated funnle, appeared two layers (oil layer and water layer), removed the water layer and collected the oil layer, the oil layer was dried over anhydrous sodium sulfate, weighed, and kept in dark sealed vials at 4 °C.

Isolation using hexane: 50 g of *ficus carica* fruit powder was added in the round flask, followed by 500 mL of hexane, and distilled in a Clevenger apparatus for 3 h. The isolation processed during three hours of distillation. The essential oils were isolated using separated funnel (as mentioned by using water solvent), weighed, and kept in dark sealed vials at 4 °C. Collected 1 mL of FCFW and FCFH from each isolation step. The percentage yield of essential oils was estimated following the equation below:

$$\boldsymbol{Essential\ oil}\ (\%) = \left[\frac{Weight\ of\ essential\ oils\ (g)}{Weight\ of\ FCF(powder)(g)}\right] \times 100$$

Gas chromatography-mass spectrometry analysis

Gas chromatography–mass spectrometry (GC-MS) was carried out utilizing a gas chromatograph connected to an Agilent 7890B GC system with a 5977A MSD and Mass Hunter workstation software. A linear gradient was obtained by rising the temperature from 50 to 280 °C at a rate of 10 °C/min in a 5 % phenyl methyl siloxane column at 6.0799 psi pressure and a column temperature gradient of 40 °C. The injector was kept at 290 °C, and the solvent was turned off after 4 minutes. At a flow rate of 1 mL/min, helium was used as the carrier gas. The injection was pulsed splitless, with a molecular weight test range of 35–650 m/z and a test rate of 1562. 1 µL of essential oils was injected into the GC column and analyzed using the National Institute of Standards and Technology (NIST) library**[24]**. The components of the essential oil were identified by comparing their Kovats retention indices to those in the literature. The Kovats index was calculated using a linear interpolation of the retention duration of a homologous series of n-alkanes (C6-C20) under identical operating conditions. Furthermore, the components were identified by comparing each

constituent's mass spectra to those held in the NIST databases, as well as mass spectra from the literature. The normalization approach was used to obtain the composition percentage of the oils from the GC peak regions, which were calculated as the mean of three samples with no correction factors**[25]**.

MTT assay

To determine the IC_{50} of essential oils, the MTT test was performed on 96-well plates. The HepG2 cell lines were planted in wells at a density of $1x10^4$ cells. After 24 hours of incubation, the cells were treated with different concentrations (10-60) %v/v, and the effect was studied for 72 hours. The growing medium was removed and replaced with 28 µL of MTT solution, which was incubated for 2 hours at 37 °C. After extracting the MTT solution, 100 µL of DMSO (Dimethyl Sulfoxide) was given to the cells, which were then incubated at 37 °C for 15 minutes. Using a micro-plate reader, the absorbance was measured at 620 nm, and the cell viability percentage was determined based on the concentrations utilized**[26,27]**.

Flow cytometry analysis

Cell Cycle arrest

Flow cytometry research has defined the G1, S, and G2 phases. HepG2 cells were subjected to essential oil IC_{50} values for 48 hours. The cells were rinsed in PBS before being fixed overnight in 70% ice-cold ethanol at 4 °C. Cells were stained for 30 minutes in the dark at room temperature with a solution containing 50 mg/mL PI and 100 mg/mL RNase A after being washed twice with PBS. Flow cytometry (partec; model: CyFlow Space; built in: Föreningsgatan 217, Landskrona, Germany; software: FLOWMA) was used to observe the labeled cells**[28].**

Reactive oxygen species

The alterations in intracellular reactive oxygen species formation were measured by labeling the cells with 2,7-dichlorfluorescein-diacetate (DCFH-DA). In 6-well culture plates, the cells were incubated overnight. Following a 48-hour treatment with IC_{50} values of oils, the cells were treated for 30 minutes at 37 °C with 10 mol/L DCFH-DA, as indicated by the manufacturer. The cells in the positive control group were tagged with DCFH-DA and rose for 30 minutes after being treated with 1 µL. Cells were then collected, washed, and resuspended in PBS before being flow cytometrically examined for 2,7-dichlorfluorescein (DCF) fluorescence**[29].**

Apoptosis

The late apoptosis was classified by flow cytometry. HepG2 cells were treated for 48 hours using essential oil IC_{50} values. The cells were collected, washed twice with PBS, and tagged with 5 µL FITC-conjugated annexin V, as directed by the manufacturer. After being incubated in the dark for 10 minutes and then labeled with PI, the samples were immediately examined on a flow cytometery**[29]**.

Table (1) GC-MS study of essential oils isolated from *ficus carica* fruit using water as extraction solvent. Main seven known chemicals were used as chemical components

List no.	Compound name	RT	RI	Concentration %	Class of compound	Formula
1	Oxime-, methoxy-phenyl-_	9.573	802	2.18	Nitrogen compounds	$C_8H_9NO_2$
2	1,1,1,3,3,3-Hexafluoro-2-(1-methoxycarbonyl)-ethoxyiminopropane	12.610	784	0.67	Nitrogen compounds	$C_7H_7F_6NO_3$
3	2,5-Dihydroxybenzaldehyde, 2TMS derivative	13.172	1322	1.69	Aldehyde	$C_{13}H_{22}O_3$
4	Benzoic acid, 3-(pentafluoropropionyloxy)-, TBDMS derivative	15.286	1687	0.61	Acid	$C_{16}H_{19}F_5O_4$
5	benzaldehyde, 4-(dimethylamino)-2-hydroxy-	17.416	955	1.58	Aldehyde	$C_9H_{11}NO_2$
6	1(2H)-Naphthalenone, 3,4-dihydro-3,3,6,8-tetramethyl-	20.101	1394	0.24	Ketone	$C_{14}H_{18}O$
7	2,5-Dihydroxybenzoic acid, 3TMS derivative	22.985	1979	0.20	Acid	$C_{16}H_{30}O_4$

RT= Retention time; RI= Retention index

Results and discussion

GC-MS analysis of essential oils components

Plant materials extracted using various solvents and extraction techniques will provide bioactive chemical libraries. The identification of bioactive compounds is crucial for establishing an understanding of the types and structures of medications that could be used to treat disorders. GC-MS is most likely the most successful multidimensional approach for essential oil analysis and identification**[30]**. Using equation (1), the essential oil percentage for FCFW and FCFH was same after three hours of extraction, and the essential oil percentage was 2%. As a result, in this investigation, we used GC-MS to detect essential oils isolated by hot distillation utilizing aqueous and hexane solvents. As shown in **Table** 1, we observed

main seven known compounds by NIST library in the FCFW essential oils. Among them, there are four compounds has the highest area, three peaks of Oxime-, methoxy-phenyl (RT=9.573 min; area%= 2.18), Phenyl - pentamethyl - disiloxane (RT=10.41 min; area% = 3.46), 2,5-Dihydroxy-benzal-dehyde, (RT=13.17 min; area%=1.69), benzaldehyde, 4-(dimethylamino)-2-hydroxy- (RT=17.416 min; area%=1.58). The essential oils of FCFW belongs to chemical classes (nitrogen, aldehyde, acid, and ketone compounds).

In FCFH essential oils, identified main two known compounds by NIST library, Oxime-, methoxy-phenyl (RT=9.523 min; area%= 2.25), and Phenyl-pentamethyl-disiloxane (RT=10.42 min;area%=2.14), as shown in **Table** 2. The essential oils of FCFH belongs to chemical classes (aldehyde, and nitrogen compounds). The retention index (RI) of theidentified compounds were determined using the Kovats method with alkanes (C6-C20) as standards.

List no.	Compound name	RT	RI*	Concentration %	Class of compound	Formula
1	Oxime-, methoxy-phenyl-_	9.523	899	2.25	Nitrogen compounds	$C_8H_9NO_2$
2	2,5-Dihydroxybenzaldehyde, 2TMS derivative	13.172	1322	0.75	Aldehyde	$C_{13}H_{22}O_3Si_2$

Table (2) GC-MS study of essential oils isolated from *ficus carica* fruit using hexane as extraction solvent. Main two known chemicals were used as chemical components.

RT=Retention time; RI= Retention index

Anticancer evaluation

MTT assay

The MTT assay was used to test the anticancer activity of essential oils isolated from *ficus carica* fruit using water and hexane solvents. The prescreening of essential oils against HepG2 cells to determine cell viability revealed that FCFW had a cell viability of 25%, whereas FCFH had a cell viability of 39.6%. These findings pave the way for estimating the IC_{50} value of both essential oil extracts. IC_{50} values were computed using excel program, and different volumes of essential oils (10-60) %v/v were utilized to analyze the relationship with cell viability. The results showed that the IC_{50} value for both FCFW and FCFH was around 40% (v/v%), as shown in **Figures** 1A and B. To target the difference in IC_{50} efficacy, we performed flow cytometry analysis to differentiate the acceptable essential oils of *ficus carica* for future investigations against HepG2 cancer cell utilizing three experiments cell cycle, reactive oxygen species, and apoptosis .

Flow cytometry analysis

Cell cycle arrest

The cell cycle is a four-step process that occurs inside the cell to understand the action and mechanism of plant extract (G1, S, and G2). This procedure will result in a checkpoint where plant extract may have a role**[31]**. To target the checkpoint, we employed IC_{50} value of FCFW in this study. We did not notice or target the proper checkpoint in FCFH because the levels of G, S, and G2 in untreated cells (control) were higher than in treated cells (**Figure** 2A and B). While FCFW showed that G1 (58.64) and G2 (16.82) in control were higher than G1 (17.05) and G2 (2.19) in treated cells, and S phase in control (25.41) was lower than S phase (34.42) in treated cells, showed that essential oils of water extract arrested HepG2 cells in S phase, **Figure** 2A and C.

Reactive oxygen species (ROS)

We used flow cytometry to examine the ROS results using the IC_{50} value of FCFW and FCFH. By comparing treated cells to untreated cells, DCFH has been frequently utilized as a biomarker for ROS. It's a dye that detects hydroxyl, peroxyl, and other ROS activity in cells. Cellular esterases then deacetylate it to a non-fluorescent molecule, which is then oxidized by ROS to 2', 7'-dichlorofluorescein (DCF). Despite the fact that DCFH is easily impacted by light irradiation during measurements, it is still a significant approach for determining ROS levels in treated cells and comparing them to untreated cells to assess the efficiency of medications or plant extracts because ROS play a role in cell damage**[32,33]**. The results showed that untreated cells (control) had 98.7 % DCFH+, FCFH had 86.5 % DCFH+, and FCFW had 35.4 % DCFH+, as shown in **Figure** 3A, B, and C.

Apoptosis

To increase the anticancer activity of *ficus carica* fruit essential oil and link the results to the cell cycle and ROS. We performed an apoptotic assay to

analyze and differentiate the efficacy of FCFW and FCFH against HepG2 cells. Apoptosis is a type of controlled cell death that occurs in eukaryotes, including humans. It occurs in both unicellular and multicellular eukaryotes, and some apoptotic pathways have been seen in bacteria. When apoptosis begins, a membrane permeability change occurs, which is defined by the collapse of the inner mitochondrial transmembrane potential. The following stage is distinguished by chromatin condensation and nuclear fragmentation. The cell then fractures into membrane-bound, ultrastructurally well-preserved fragments that are swallowed by macrophages, preventing inflammation from being induced**[34,35].** We employed the identical FCFW and FCFH IC_{50} values to identify early apoptosis (Q1), late apoptosis (2), necrosis (Q3), and cell survival (Q4) (Q4). **Figure** 4B shows that treated cells with an IC_{50} value of FCFH showed early apoptosis (43.1%), late apoptosis (24.3%), necrosis (6.88%), and alive cells (25.7%). **Figure** 4C shows that treated cells with an IC_{50} value of FCFW showed early apoptosis (12.7 %), late apoptosis (30.2 %), necrosis (17.0 %), and living cells (40.0 %). When treated cells were compared to control cells, the efficacy of apoptosis results indicated early apoptosis (9.16 %), late apoptosis (2.44 %), necrosis (2.49 %), and lives cell (85.9 %), as shown in **Figure** 4A.

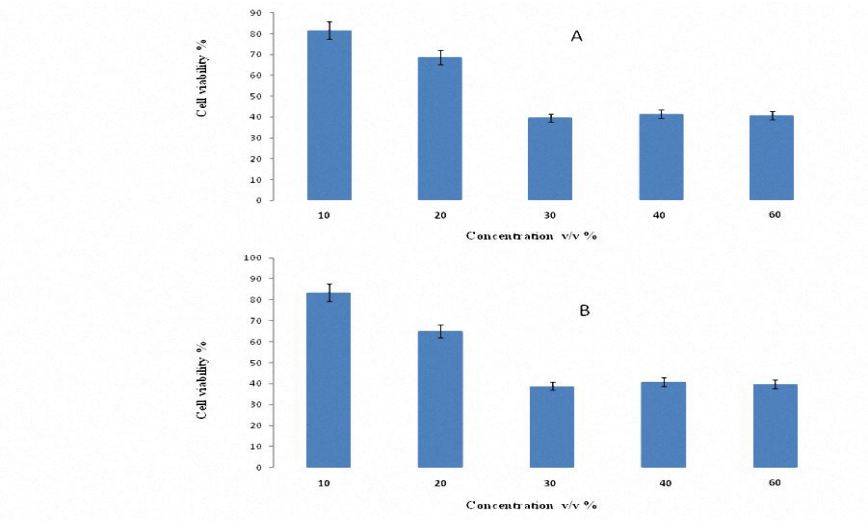

Figure (1) MTT test of *ficus carica* fruit essential oils at various doses in order to estimate the IC_{50} value: **A-** Essential oil isolated using FCFW. **B-** Essential oils isolated using FCFH.

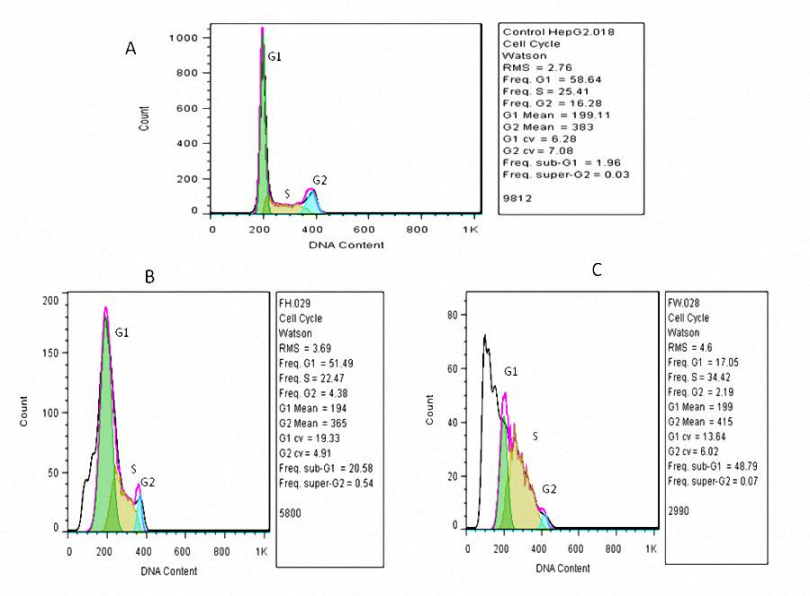

Figure (2) Flow cytometry analysis of *ficus carica* fruit essential oils, showed the cell cycle of: A- untreated cells. **B-** Cells treated with IC_{50} value of FCFH essential oils. **C-** Cells treated with IC_{50} value of FCFW essential oils.

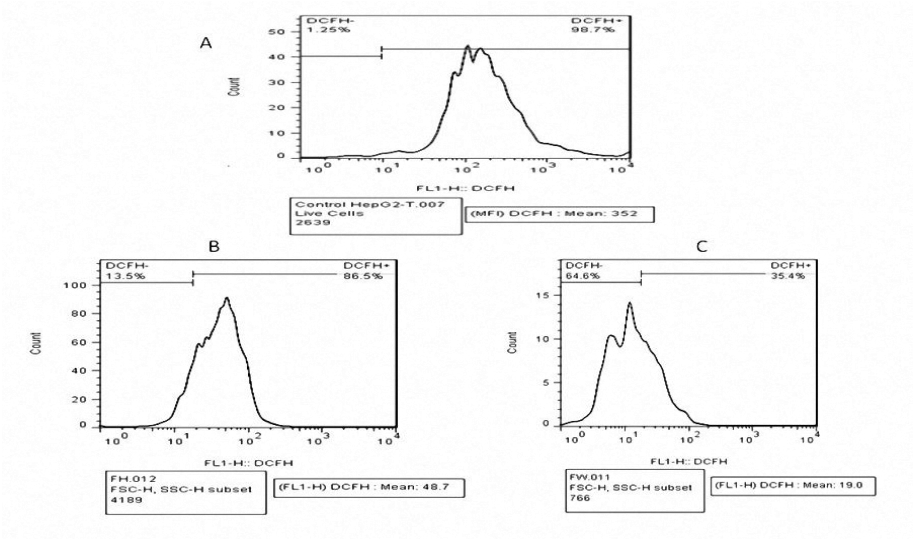

Figure (3) Flow cytometry analysis of *ficus carica* fruit essential oils, showed the reactive oxygen species of: **A**- untreated cells. **B**- Cells treated with IC_{50} value of FCFH essential oils. **C**- Cells treated with IC_{50} value of FCFW essential oils.

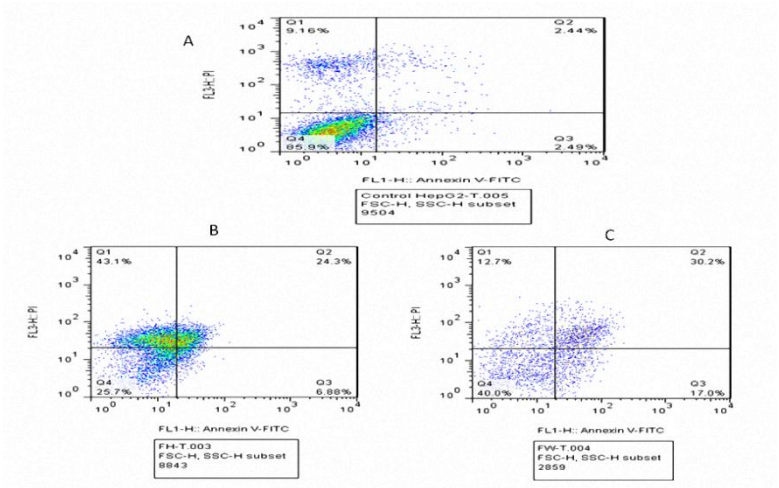

Figure (4) Flow ctytometry analysis of *ficus carica* fruit essential oils, showed the apoptosis of: **A**- untreated cells. **B**- Cells treated with IC_{50} value of FCFH essential oils. **C**- Cells treated with IC_{50} value of FCFW essential oils.

Essential oils have recently gained prominence in biological applications such as antibacterial, antioxidant, anti-inflammatory, antiviral, and anticancer properties**[36-38]**. The principal ways by which essential oils mediate their cytotoxic effects include the stimulation of cell death by activation of apoptosis and/or necrosis processes, cell cycle arrest, and loss of function of key organelles**[39]**. According to the literature, *ficus carica* extract and latix have strong apoptosis inducers on human colorectal cancer cells. Soltan et al., shown that *ficus carica* extract and latix had high apoptosis inducers on human colorectal cancer cells**[40].** Zhang et al. demonstrated that *ficus carica* leaf extract could be a promising source for developing medications to reduce cancer-cell proliferation and migration in the treatment of triple-negative breast tumors**[41].** Purnamasari et al. discovered that when compared to fruit extracts, methanol extract of *ficus carica* leaf showed a higher proportion of liver cancer cell (Huh7it) apoptosis and necrosis**[42]**. Thus, GC-MS analysis, antioxidant, and antibacterial activity of *ficus carica* leaf essential oils were demonstrated in a study by**[43].** To the best of our knowledge, no one has reported on the essential oils of *ficus carica* fruit, chemical composition analysis, and anti-liver cancer activity. In this study, we used *ficus carica* fruit to isolate essential oils using water and hexane solvents, characterized the chemical compositions using GC-MS, and examined their anticancer activity by MTT

assay, cell cycle, ROS, and apoptosis using HepG2 cells for the first time. The GC-MS analysis of FCFW showed sixteen chemicals, with the majority of them belonging to the organosilicon (9), nitrogenous (2), aldehyde (2), acid (2), and ketone (1) classes of compounds. Organosilicon (9), aldehyde (1) and nitrogen (1) compounds account for ten of the FCFH chemicals. These chemical families in essential oils have been linked to pharmacological uses such as antioxidant, chronic inflammation, anticancer, and antibacterial properties**[44-48]**. The next step was to investigate the anticancer effects of FCFW and FCFH essential oils. MTT assay was used to measure the IC_{50} value, and the essential oils FCFW and FCFH have similar IC_{50} values. To determine the cell cycle phase, we employed the IC_{50} value for essential oils of FCFW and FCFH. For FCFW, there were clear differences in the G1, S, and G2 values of treated cells compared to the control and arrested cells in S phase, whereas FCFH could not recognize the detected cell cycle phases of treated cells, which could be attributed to the compounds combined functions in the FCFW and FCFH. Furthermore, the ROS experiment revealed that FCFH essential oils lowered than FCFW essential oils, and FCFW late apoptosis was higher than FCFH late apoptosis. Our findings show that essential oils of FCFW are more effective than essential oils of FCFH in arresting the cell cycle in S phase, lowering ROS and late apoptosis in HepG2 cells. As a result, it has improved the medical application of essential oils produced from FCFW and plans to expand its application in the future research.

Conclusion

Because of its advantages over other synthetic medications, the usage of essential oil has grown dramatically in recent years. To meet human requirements, essential oils have been employed for a variety of applications, including aromatherapy, food, flavoring, and medications. Essential oils include unique and powerful anticancer chemicals that can be utilized in cancer treatments.

In the present study, essential oils isolated from *ficus carica* fruit using water and hexane solvents, were tested against human liver cancer cells. The anticancer evaluation revealed that *ficus carica* fruit essential oils isolated by water might be developed through future research in the prevention of liver diseases and other diseases.

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