How to Cite:
Mohammed, S., & Habeeb, H. M. (2022). Phytochemical and cytotoxic study in Quisqualis indica volatile oil cultivation in Iraq. *International Journal of Health Sciences*, *6*(S4), 8572–8584. https://doi.org/10.53730/ijhs.v6nS4.10618

Phytochemical and cytotoxic study in Quisqualis indica volatile oil cultivation in Iraq

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***Abstract*---**The volatile oil is metabolites product from some plant have biological activities such as antimicrobial ,antioxidant and have viability for reduced cancer cell ,through inhibit cell proliferation ,for this reason This study was set to extraction volatile oils from Inflorescences of Qusiqualis indica plant cultivation in Iraq from tow season (spring ,autumn)and identification of the Chemical compounds of these (using GC-MS) ,The cytotoxic effect of two seasons volatile oils on human lung cancer cells A549 compared with normal REF cell cells were studied at concentrations (5,6,12,25,50)µl/ml and the programmed cell death (apoptosis) tested for cells lines and measured P53 gene ,Caspase-8 expression levels. The results showed significant quantitative and quantitative in two seasons oils ,and there were significant differences in the degree of cytotoxicity between the two oils ,where is at the concentration (50 µl/ml)they have the highest effects of(A549)cells compared with REF cells ,while the higher apoptosis effect at (25 µl/ml) concentrate for A549 compared with REF.

***Keywords*---**volatile oil, Quisqualis indica, A549, cytotoxicity, apoptosis, caspase-8, P53.

Introduction

Volatile oil or essential oils are plant metabolic compounds that are naturally produced by aromatic plants, either to protect against predators or pollinators for seed dispersal, in addition to giving the plant a distinctive character by its smell (12).The volatile oils contain many compounds and the basic unit of V.O is terpene, which have volatile properties because they contain oxygen in their

chemical composition, in addition to the carbohydrate chain or ring depending on the type of oil, in the essential oils may be in the form of phenolic compounds (19). Despite the characteristics of oils and their function for the plant body, they are characterized by many medicinal qualities, as they have proven their effectiveness as an antioxidant, anti-cancer, anti-microbial, and diabetes and for kidney disease in laboratory animals , in addition to their use in the manufacture of natural cosmetics and perfumes (3,14). There are more 3000 essential oils from more than 2000 plant species, and 300 of these oils have many medicinal and biological properties(15). Volatile oil examed as anti cancer in ivivo or in vitro and there activity for programmed cell death and gene expression (24)according to (29) were study the essential oils differ according to the geographical location, cultivation conditions and the season of plant collection to obtain the volatile essential oil.

Qusiqualis indica is home ornamental plant member of Combretaceae Asian family also called Rangoon creeper and red jasmine ,have inflorescence type receme and this flowers is regular pentamerous petals, it's have many properties for medicinal uses in folklore herbal medicine such as anthelmintic, heal boil ,ulcers and ringworm(22).It's have many phytochemical active compounds such as(alkaloids ,flavonoids ,terpene ,saponins ,steroids ,phenolic compounds ,tannins and many fatty acid ,essential oils) (17,11).

Cancer is the second leading cause of death in the world,Cancer is a major public health problem both in developed and developing countries around the world. It is estimated that there were (13,29). million recent cancer cases and about 10 million cancer deaths in 2020, which reflects the harmful impact of cancer on humans of all kinds, In fact, most synthetic chemotherapy drugs currently used in cancer treatment are toxic and damage normal cells, Therefore chemoprevention or chemotherapy via non-toxic agents can be the solution to chemotherapy problems (27) According to the World Health Organization .Lung cance, is the most common cancerous disease that causes death in smokers that suffer from it, its risk increases with age , smoking and its risk increases in males more than females, The death rate due to lung cancer constitutes 25% of all infections, while 71% of lung cancer deaths are Of smokers and the rest of the injuries caused by exposure to radioactive substances such as radon and environmental pollutants such as arsenic and silica(21).

The programmed cell death(apoptosis)which is the process cell kills itself programmatically according to a series of cellular processes and without secreting harmful substances in the surrounding area. This process leads to the disposal of old cells, unnecessary cells and sick cells which keeps the body healthy.) At the same time that the cell death process is replaced by new cells, the imbalance in the death of old cells and the production of new cells may cause a disorder in the balance of these cells and thus develop many diseases such as cancer and neurodegenerative disorders such as Alzheimer's disease and the process is controlled by many proteins including the Caspase group and P53 gene.(21) The Volatile oils of *Qusiqualis indica* is firstly extracted in Iraq ,only two studies extracted and examined the active compound in oils in India but the results it's more different between Iraq and India,so we studied effects of volatile oil on lung

cancer especially because the oil can inhaled and easy treated with respiratory diseases.

Materials and Methods

Plant materials

Plant collection and classification

(Flowers,inflorescences) were collected from agricultural nurseries and home gardens in Baghdad city Iraq, and throughout the spring and autumn seasons, where the spring flowers were collected from mid-April to the end of June, and the autumn flowers were collected from mid-September to mid-November**.**

Volatile oils extraction

The volatile oil was extracted by a Clevenger type apparatus, where extraction was done by hydro distillation technique, the fresh flower extracted with distilled water the ratio (1:5)v/v for (13) hours continue at 50-70 ,the percentage of V.O measured according to the following equation.(25)
Percentage of Essential oils=volume of oils was extracted/ weight of whole plant.

Quantitative and qualitative analysis of volatile (essential)oils

The components of the two seasons essential oil of the plant were identified by a gas chromatography-mass spectrometer (GC-MS) type (A USA GC-ms 7820 Agilent).

Cell growth preparation

Cytotoxicity assays

The lung cancer A549 cell line and the normal cell line Rat embryo fibroblast REF cell were treated with Volatile oil to determine the cytotoxic effect of Volatil oils with MTT assay using 96-well plates(4).The cytotoxicity effectiveness were examined for three time (24,48,72)h.at (50,25,12.5,6.25) µl/ml concentration and incubation at 37C ,the inhibitors rate were measure using the following equation(7,9,5,8).
Inhibition rate = A- B/A*100
where A is the optical density of control, and B is the optical density of the samples (18)

Apoptosis assay

Acridine Orange–Ethidium Bromide Staining

The Volatile oils induced death of A549 cells was assessed using the AO/EtBr (Sigma–Aldrich, USA) staining method using (100µl)of a Dual fluorescent dyes. After 24 h. the seeding of cells in 12- well plates, they were treated with Volatile

(essential) oils and incubated for an additional 24 hr.,. Finally, the cells were observed using fluorescence microscopy(6,26).

Measurement of Caspase-8 and p53 Levels(Flow Cytometry Assay)

The activation states of caspase-8 and p53 were evaluated using a fluorescein caspase-8 and p53 staining kit (Thermo Fisher Scientific, USA), The prepared cells A549,REF were then incubated with 1 mL of FITC-IETD-FMK at 37°C for 60 min(16).

Statistical analysis

The obtained data were statically analyzed using an unpaired t-test with Graph Pad Prism 6. The values were presented as the mean ± SD of triplicate measurements

Results and Discussion

The Volatile oils extracted and isolated from the two seasons were analysed using GC-ms , the results elucidate there are differences in the compounds and in the amount of the same volatile oil in the two seasons , as the volatile oil of the autumn season was have nine chemical Compounds and it's quantity was 2.5%ml, while the spring contains 18 chemical compounds, were its rate 1.5%ml ,the compounds ,percentage of each one and their proportions for the two seasons oils were found in the table and curves(figure-1)

Table 1. GC ms analysis The spring volatile oils of Q. indica

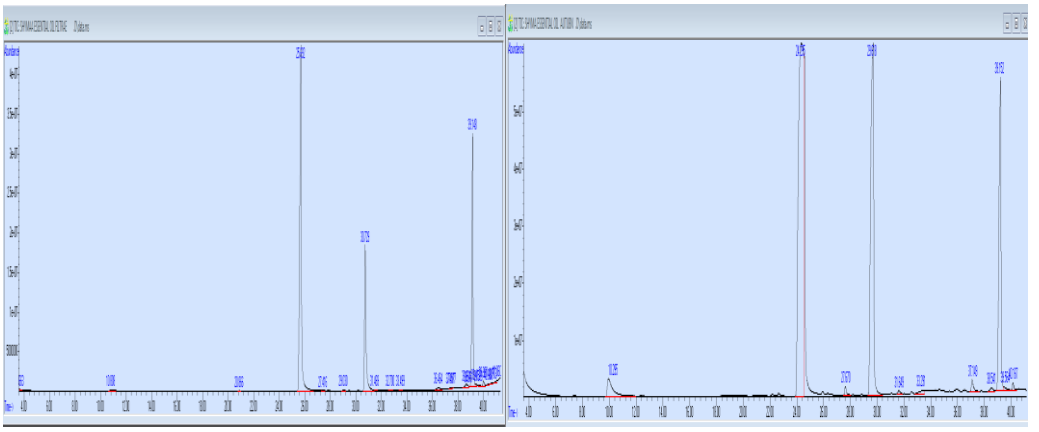
Chemical class, Synonym	retention time percentage%	Molecular weight, formula	Name of chemical compounds	Number of peak
-mono terpene -Limonene	10.808 0.59%	: C10H16 MW: 136.23g/mole	D-Limonene	1
-organic silicone and ester volatile -7Cyclomethicone	20.866 0.05%	C10H30O5Si5 MW: 370.77g/mole	Cycloheptasiloxane, tetradecamethy	2
-esters -Dioctyl ether	26.554 50.97%	C12H18OS4 MW: 306.5g/mole	Octane, 1,1'-oxybis-	3
-ester -Non	30.729 18.99%	C16H30O4 MW: 286.41g/mole	Oxalic acid, 2- ethylhexyl	4
-monounsaturated fatty acid -Vaccinic acid	32.7 0.12%	C18H34O2 MW: 282.5g/mole	cis-Vaccenic acid	5
-essential oil -Eicosene(cis/trans mixture)	33.49 0.07%	C20H40 MW: 280.5g/mole	5-Eicosene, E	6
-fatty acid ester -1-Propyldodecyl octanoate	36.464 0.64%	: C23H46O2 MW: 354.6g/mole	Octanoic acid, 4- pentadecyl ester	7
-understand fatty	37.455	C18H34O2	oliec acid	8

acids -cis-9- Octadecenoic acid	0.23%	MW: 282.5g/mole		
-aromatic -Fluorobenzene	38.664 0.87%	C7H3F2NO MW: 155.1g/mole	Benzene, 2,4-difluoro-1-isocyanato	9
-amino acid ester -L-Histidine methyl	38.851 0.08%	C12H18N4O6 MW: 314.29g/mole	Histidine,N-t-butyloxycarbonyl-4-nitro-, methyl(ester)	10
-alkene TETRAMETHYLNONADECANE	39.148 25.35%	C23H48 MW: 324.6272	Nonadecane, 2,6,10,14-tetramethyl-	11
-Ketones -non	39.412 0.24%	C38H69F7O2 MW: 690.9421	Tetratriacontyl heptafluorobutyrate	12
-ester -non	39.599 0.11%	C17H32O2 MW: 268.4g/mole	7-Methyl-Z-tetradecen-1-ol acetate	13
-ester -Ethyldodecyl trichloroacetate	39.998 0.63%	C16H29Cl3O2 MW: 359.8g/mole	Trichloroacetic acid, 3-tetradecyl ester	14
-aromatic -n-(boc-4-chloroaniline)	40.380 0.20%	C11H14ClNO2 MW: 227.69g/mole	N-tert-Butoxycarbonyl-4-chloroaniline	15
-Alkane -non	40.703 0.21%	: C19H40 MW: 268.5g/mole	5-Ethylheptadecane	16
-Alkane - non	40.933 0.20%	C18H38 MW: 254.5g/mole	Heptadecane, 4-methyl-	17
-steroidal alkaloids -Buxpsiine	41.060 0.31%	C26H39NO MW:381.59g/mole	Ba(9a)-Homo-19-norpregna-9(11),9a,16-trien-20-one, 3-(dimethylamino)-4, 4,14-trimethyl-, (3beta,5alpha)	18

Table 2. GC ms analysis of the autumn volatile oils of Q. indica

Chemical class ,Synonym	retention time, percentage%	Molecular weight, formula	Name of chemical compounds	Nomber of peak
-mono terpene -limonene	-10.293 -2.91%	C10H16 MW: 136.23g/mole	D-Limonene	1
-esters -Di Octyl ether	-24.295 -48.8%	C12H18OS4 MW: 306.5g/mole	Octane, 1,1'-oxybis-	2
-ester -non	-29.156 -29.25%	C20H38O4 MW:342.5 g/mole	Oxalic acid, decyl 2-ethylhexyl ester	3
-fatty acid ester -Methyl petroselinate	-31.650 -0.23%	C19H36O2 MW:296.5 g/mole	Octadecenoic acid, methyl ester	4
-understand fatty acids -cis-9- Octadecenoic acid	-33.291 0.70%	C18H34O2 MW: 282.5g/mole	Oleic Acid	5

-saturated fatty alcohol -non	-37.146 -0.65%	C19H36O MW:280.5g/ mole	2-Methyl-Z,Z-3,13-octadecadienol	6
-aromatic -Fluorobenzene	-38.540 -0.31%	C7H3F2NO MW: 155.1g/mole	Benzene, 2,4-difluoro-1-isocyanato	7
-triterpene -Spinacene, Supraene.	-39.150 -16.82%	C30H50 MW: 410.7g/mole	Squalane	8
-fatty alcohol -hexahydrofarnesol	-40.168 -0.33%	C15H32O MW: 228.41g/mole	1-Dodecanol, 3,7,11-trimethyl-	9



A. **B**

Fig.1 curve of volatile oils in GC-MS data (A-spring,B-autumn)

The volatile oils of *Quisqualis indica* is firstly extracted in Iraq ,only two studies extracted and examined the active compound in oils in India but the results it's more different between Iraq and India the major components in subcritical CO2-extract of the inflorescence Indian studies to be the linalool oxides (furanoid and pyranoid) (2,2,6-trimethyl-6-vinyl-3-oxo-tetrahydropyran, (E,E-alpha-farnesene,(Z)-3-hexenyl benzoate and benzyl benzoate(23).In this study the Clevenger-extract and examined the compound in GC-MS make more varieties in results ,the major chemical active compounds in Iraqi oils two seasons[Octane, 1,1'-oxybis- Oxalic acid, decyl 2-ethylhexyl ester, D-Limonene,Octanoicacid, 4-pentadecyl ester, 6-Octadecenoic acid, methyl ester, Oleic Acid,Benzene, 2,4-difluoro-1-isocyanato,,extra],the high concentrate in chemical compounds in [Octane, 1,1'-oxybis was in autumn oils 48.8% but in spring oil 50.97%,Nonadecane, 2,6,10,14-tetramethyl- in spring oils 25.35%, in autumn oil Oxalic acid, decyl 2-ethylhexyl ester 29.25%],the different between two seasons compounds such as (Squalane) in autumn,(Buxpsiine) in springs .Probably these difference can be attributed to the volatile oils differ according to the season of plant collection to obtain the oil (29).

Cytotoxic effects of *Quisqualis indic* two volatile oils

Cytotoxic effect of the volatile oil extracted from *Q. Indica i*nflorescence was examined on the lung cell line (A549)and Normal cell REF at different concentrations ranging from (6.25,50µl/ml) by four dilutions the results presented that have all concentrations cytotoxicity against lung cancer cell line and safety to REF cell for three exposure time,while the inhibitory rate decreases as the concentration and incubation time decreased.

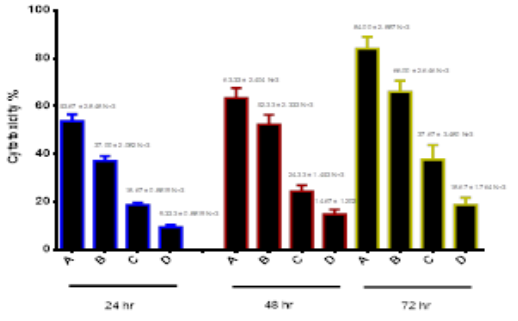
Cytotoxic effects two Volatile oils on A549 cell line

The inhibitory effect of the spring oil showed in (fig,2,3)after 24h of treatment show that(50µl/ml) was highest inhibitory rate(53.67%),where lowest inhibitory cell rate in concentration (6.25µl/ml) is (9.33%), compare with REF cell the highest inhibition viability cell rate(5.667%)and the lowest rate (1.667%).At 48h incubators treatment with spring oil the highest inhibitory rate (63%) was observed when using the concentration 50µl/ml and lower inhibit cell proliferation in concentration 6.25µl/ml percentage (14.67%) ,compared with REF cell higher inhibit cell rate(8.333%),lower inhibit cell rate (3%).After 72h treatment with spring oil was clear at the concentrations 50µl/ml, were gave the highest percentage of inhibitory rate (84%),where lowest percentage of inhibit cell viability is (18.67%) in 6.25µl/ml concentration,compared with REF cell the highest inhibitor is 9% in 50µl/ml and lower inhibit cell in (6.25 µl/ml) the percentage is 3.333%.

While the effects of autumn volatile oil exposure results show that in(fig.4,5)the less cytotoxicity level was recorded at autumn oil concentration (6.25 µl/ml) after 24h incubation period giving, the cytotoxicity level of inhibit A549 cell is (5.667%),and highest level of inhibition A549 cell in concentration 50µl/ml after 24 of treatments (48.67%),compared with REF cell is higher inhibit cell in concentration (50µl/ml) range in(5.667%) and lower inhibit cell in concentration (6.25µl/ml) is(2%).

In48 incubation and treatment with autumn oils the highest inhibit cell of A549 is 57.33% in concentration(50µl/ml)and lowest level of inhibit is 11.33% in (6.25 µl/ml),while the treatment of REF cell higher of cell inhibit (7.667%) in concentration (50µl/ml) and lower inhibit cell in concentration (6.25µl/ml) is (3.667%).The best cytotoxicity treatment in autumn oil at 72h treated have(78.67%) inhibits cell viability at(50µl/ml)concentrate,lowest percent of inhibit cell viability is(14%),compare with REF cell in same concentrates the the inhibition rate is (9.33%) and (4%).

The best treatment for inhibit cell viability with spring oil in 72 hour incubation for A549.



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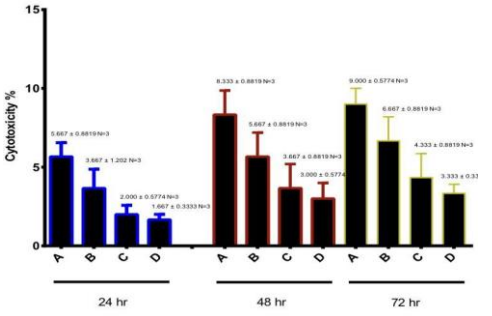
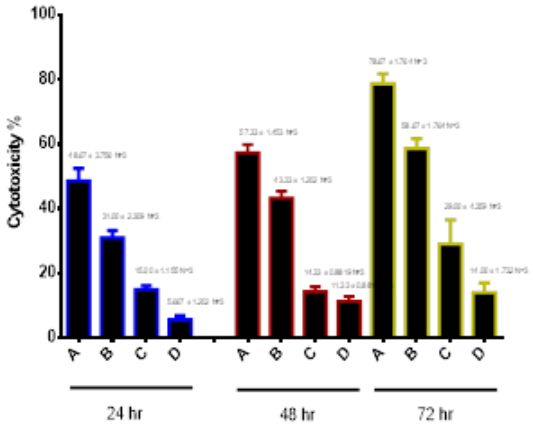


Fig:2 Cytotoxic effect of Spring in A459 cells./. Fig:3 Cytotoxic effect of Spring in REF A, B, C, D, Represented samples diluted. // cells. A, B, C, D, Represented samples diluted (50,25,12.5,6.25) respectively samples diluted (50,25,12.5,6.25)respectively



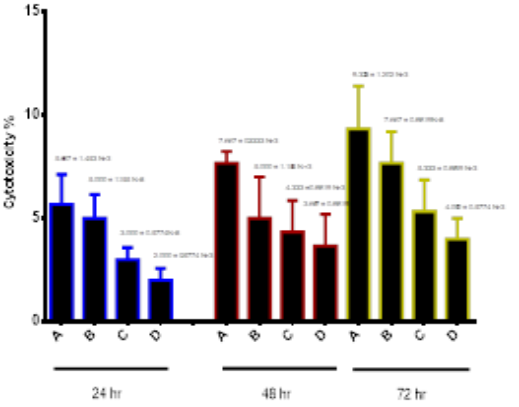


Fig:4 Cytotoxic effect of Autumn in A459 cells/ Fig:5 Cytotoxic effect of Autumn in . A, B, C, D, Represented samples diluted/ REF cells. A, B, C, D, Represented samples diluted (50,25,12.5,6.25)respectively./diluted (50,25,12.5,6.25) respectively

Apoptosis activity in two Volatile oils of Q.indica

Acridine Orange–Ethidium Bromide Staining

The results show in Figure:(6,7) the pictures of fluorescence microscopy for A549 cell and REF after 24h incubation at (25µl/ml)concentration, the pictures show (yellow and red colour) that indicate the inhibitor or death cell where is the greenish one indicate the proof viable cells.

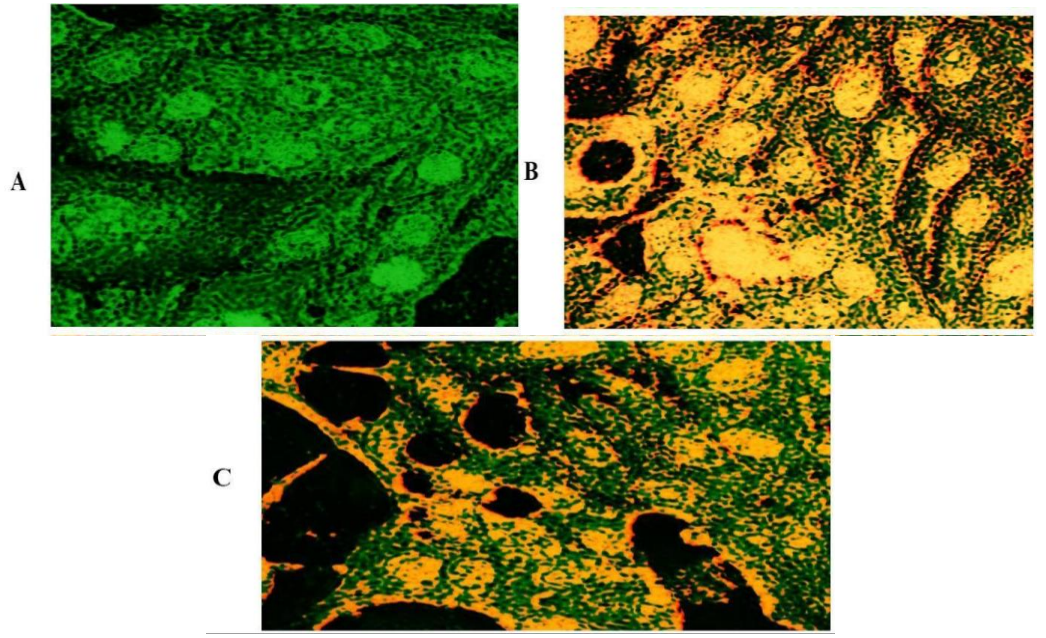


Fig:6. Apoptosis marker in A549 cells after treatment with Spring and autumn . A, represented control untreated cells, B, cells treated with Autumn C, cells treated with Spring

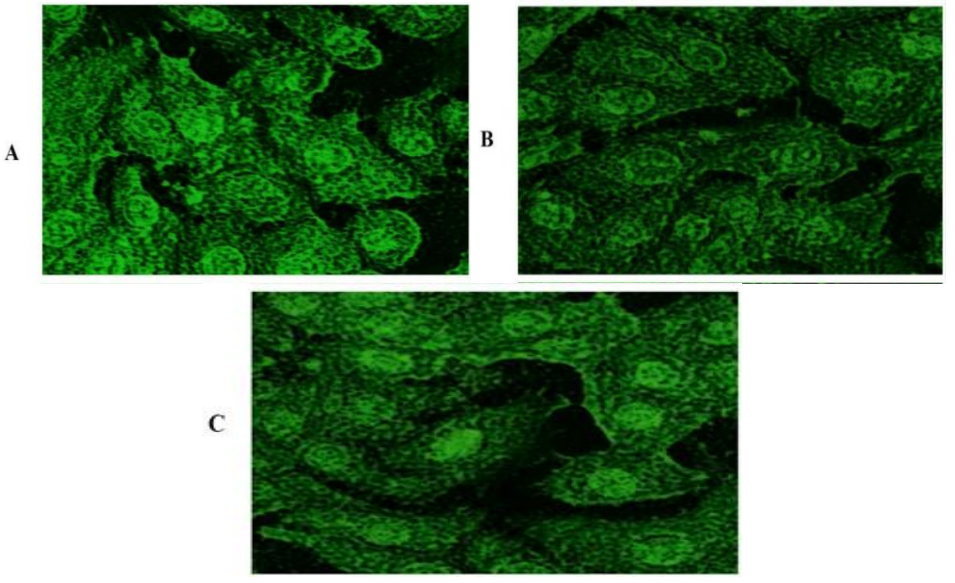


Fig:7..Apoptosis marker in REF cells after treatment with Autumn and Spring . A, represented control untreated cells, B, cells treated with Autumn C, cells treated with Spring

P53 gene and Caspase-8 levels

The results demonstrated that the cells treated with Spring oils and Autumn exhibited a marked shift toward the right for both caspase-8 and p53(fig.-8), which were designated as the apoptosome. Apoptosomes can be observed in apoptotic events, leading to the systematic disassembly of the cell.Experience , measured the levels of p53 mutation and caspase-8 expression. The results presented that treatment of A549cells with the test compounds led to a significant upregulation of both P53 and caspase-8 compared with control,wereis the spring volatile oils expressed the caspase-8 and P53 more than autumn.

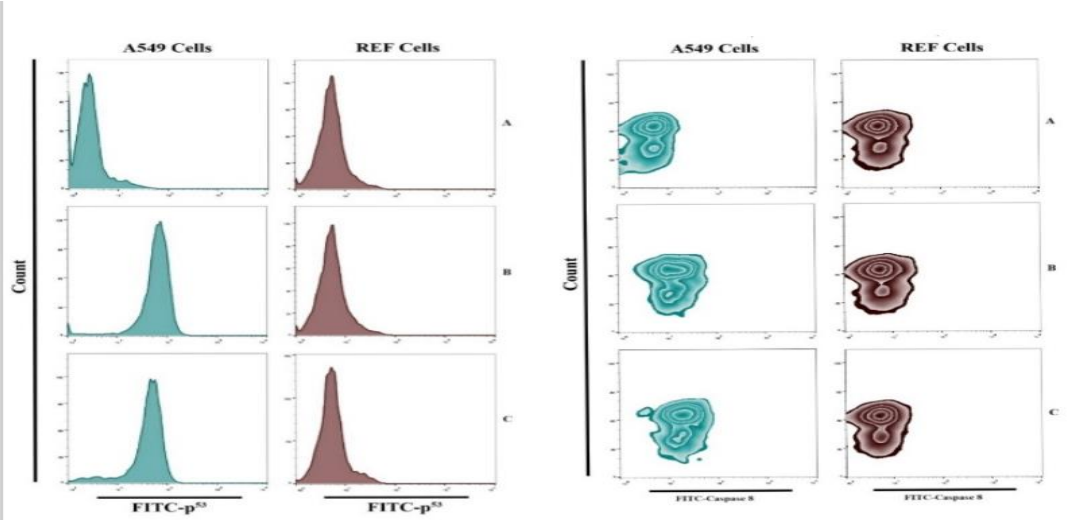


Fig:8.GRAY AND RED up regulated of P53 and Caspase-8 level in A549 cells.A, represented control untreated cells,B,clles treated with GRAY , C,cells treated with RED

The Q. indica L. has been proven to be anticancer or antitumor activity and apoptosis effects , which the anticancer activity have been demonstrated in number of studies of inflorescences extraction, particularly(20),show Anti-inflammatory and antioxidant exhibit in volatile oil of Q.indica which extracted by supercritical Co2 technique(23).Octane, 1,1'-oxybis which is highest percentage in to oils that many studies prove its have anti-cancer and Alzheimer particularly (28).In this study the Qusiqualis indica L. Volatile oils inhibited human lung cancer cell The cytotoxic effect of the Q. Indica volatile oil on the lung cancer cell line A549 perhaps is due to the present (Octane, 1,1'-oxybis-) which is the major component that may inhibits the cancer cell proliferation without affecting the normal cell and program cell death apoptosis in addition to others compounds in the oils. Where is the result that spring volatile oils higher cytotoxicity than autumn volatile oil also increased expression and apoptosis levels.These results agree with the feedback of (1)who found that the anti- proliferative activity of the oil extract Lemon grass showed that growth on A549 Cells was inhibited by the essential oils extract at concentrate(IC50, 29.13 ppm) had significant higher expression (P≤ 0.05) compared with untreated cell changes of folds is 4.47±.A study done by(2) the oxalic acids (which is contain of two oils)provide the body

calcium regulate in blood in low concentrate, while in higher concentration provided anti microbial ,antioxidants and anti cancer and safety in normal cell perhaps the cytotoxicity of *Q.indica* two oils is due to contain oxalic acid. In the study antiproliferative effect of *Q.indica t*wo volatile oils on lung cancer cell A549 and the upregulation ofP53 induce apoptosis, The p53 mediated the mitochondrial apoptosis pathway through stimulating caspase-8 activity which in turn release other apoptogenic factors such as cytochrome-C. Although many studies have examined the importance of plant extracts anticancer drugs for cancer promising treatment, they need other studies to investigations including cytotoxicity and safety in others type cancer cells(10).

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