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Antifungal Activity , GC-MS Analysis of *Thuja occidentalis* Essential Oil with Gene Expression

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Abstract: The study includes identification of Chemical compounds of *Thuja occidentalis* essential oil teste as anti- *Aspergillus flavus* isolates and expression measure the essential oil affects in aflR-2 gene expression responsible for aflatoxin production . Thirty-five compounds were identified in *T.occidentalis* EO . The result were thujone (21.58%), camphor (18.92%) and Isobornyl acetate (9.71%). The essential oil inhibited of *A. flavus* fungus at a rate of inhibition 55.5% . and decrease in aflR-2 gene expression responsible for the aflatoxin product was observed.

Keywords: *Thuja occidentalis*; Essential oil; *Aspergillus flavus*; gene expression ; aflR-2 ; aflatoxin.

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Introduction

Thuja is a small genus of the *Cupressaceae* family genus with five species, including Thuja occidentalis(1). *T. occidentalis* is also known as American Arbor vitae or white cedar. It isnativeto eastern North America and grows as an ornamental tree in Europe(2). The plant was first described by native Indians in the 16th century as a cure for treating scurvy deficiency(3). In folk medicine, *T.occidentalis* has also been used to treat bronchial catarrhal, cystitis, psoriasis, enuresis, amenorrhea, uterine carcinomas, and rheumatism(4). In addition, the leaf-isolated essential oils (EOs) have been used to treat fungus infections, cancer, moles, and parasitic worms(5).

In the last few years, most of the phytochemical studies on this plant species have centered on the terpene composition of essential oil, where α-pinene, α-cedrol, α- and β-thujone, camphor, 1,8-cineole, linalool and linalyl acetate have been identified as the major components of the leaf oil(6).

Mycotoxigenic filamentous fungi have the ability to contaminate a wide range of food and animal feed with one or more aflatoxin (7). Aflatoxins (AFAs) may be produced in food and feed during pre-harvest, drying, processing or storage. Because of the toxicity and carcinogenicity of mycotoxins, infected products

consumed by humans or animals constitute a serious health risk and are thus carefully regulated and controlled(8). Aflatoxin B1 (AFB1) is mutagenic and teratogenic, and can suppress immunity that is mediated by cells. It also plays a role in lung invasion and aspergillosis ball formation(9).

Real-time PCR is extremely sensitive, with the ability to measure unusual transcripts and minute gene expression changes. Real-time PCR used to test and track the fungus' ability to activate or inhibit the biosynthesis of mycotoxin under various antifungal genes(10, 11).

The purpose of this research was to identify the active chemical compounds in the essential oil of *T. occidentalis* and test it as anti-fungal for *A. flavus* and verify how it affects the expression of the gene aflR-2 responsible for producing aflatoxin.

Material and Methods

Essential Oil

Essential Oil *T. occidentalis* was acquired from AROMA LIFE by import.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

Essential oil chemical components of the sample were identified by a Varian GC–MS spectrometer with a fused-silica column (DB-5, 30 m 0.25 mm i.d., J and W Scientific Inc.). The oven temperature was set at 60–240 ˚C with a ramp of 3 ˚C/min, injector temperature 280 ˚C, injector mode: split injection, with He (helium) as the carrier gas at a flow rate of 2 ml/min. The mass spectra ionization potential was 70 eV, ion source temperature of 250 ˚C. The GC was a Shimadzu GC-17 equipped with a FID detector, fused-silica column (BP-5, 25 m 0.22 mm i.d.). The operating conditions were: oven temperature 60– 280˚C with a rate of 8˚C/min, injector temperature 280˚C, split ratio 1:10, with N2 carrier gas, and detector temperature 300˚C.

Antifungal Activity

Fungal isolates

Two isolates of *A.flavus* fungus were obtained from the University of Kerbala and three isolates from the University of Kufa, isolated from infected corn grain. Isolates were cultivated on the medium of Sabouraud Dextrose Agar (SDA). Then, the colony diagnosed using morphological and microscopic characteristics; examination was carried out using the lactophenol cotton blue dye (12).

All fungi isolates were maintained as slant in two replicate each on Sabouraud Dextrose Agar (SDA) composed of as stock cultures at 4-8 °C. These were reactivation by regular sub-culturing protocoled by(13).

Agar Well-Diffusion Method (ADM)

The antifungal effect of EO was examined against the fungal isolates *A.flavus*. Fungal cells were cultured on sabaroed dextrose agar (SDA) solid

media. Agar well diffusion method was followed. Wells were punched of 8 mm diameter into the SDA medium and filled with 100µl containing EO, Nystatin (**Sigma-Aldrich**) and DMSO as control (*A.flauvs* only) treatment, in different concentrations (1000000, 500000, 250000, 125000, 62500, 31250, 15.625 and 7.812) ppm (EO/DMSO), Concentrations were nystatin (1000, 500, 250 , 125, 62.5, 31.2, 15.6 and 7.8) ppm (powder nystatin/water) and incubated at 27 °C for 5 days. Controls of Silver-free plates were incubated under the same conditions.

Data analysis

The radial growth of fungal mycelium was recorded on SDA plates containing EO, when mycelial growth reached the edge of the petri dish, radial inhibition was calculated. The following equation was used for the inhibition rate(%) calculation.

Inhibition rate (%) = R-r\R

Where (R) is the fungal radial growth of on the control test materials free plate and (r) is the radial of fungal growth on culture plate inoculated with EO.

Molecular study

Total RNA extraction

After 5 days of *A. flavus* incubation on SDA medium, the entire mycelial colony was removed. Total RNA were isolated using (Quick-RNA Fungal/Bacterial Miniprep Kit) according to the manufacturer's instructions (Zymo Research). The purity and concentration of the RNA was measured by a Nano Drop ND-2000 spectrophotometer (Thermo Scientific) device at a wavelength of 260 - 280 nm.

Preparation of primers

Specific primers were obtained (Table1) according to the previous studies, for detection of the gene expression.

Table (1): Sequences of primers that used to gene expression

Primers		Primer sequence	Product size (base pair)	Reference
aflR-2	F	GCACCCTGTCTTCCCTAACA	400 bp	Manonmani *et al*. (14)
	R	ACGACCATGCTCAGCAAGTA		
House-keeping gene (β-tubulin)	F	TCTTCATGGTTGGCTTCGCT	118bp	Mitema *et al*. (15)
	R	CTTGGGTCGAACATCTGCT CTTGGGTCGAACATCTGC T		

One step Quantitative Real-time PCR Assay (QRT-PCR) by using KAPA SYBR FAST one-step qRT-PCR kit-Canada. Amplification of the mRNA fragment was performed with master amplification reaction with the One-Step RT-PCR list system in Table (2) and Table (3) method. Several experiments were conducted to more appropriate cDNA synthesis and temperature annealing.

Table (2): One-Step quantitative RT-PCR Reaction Mix

Component	20 µL (Final volume)	Final concentration
Sybr green kappa Master mix	10 µL	
Forward primer	0.4 µL	0.2µM
Reverse primer	0.4 µL	0.2µM
50X KAPA RT Mix	0.4 µL	1X
Nuclease-free water	4.2 µL	
RNA Sample Volume	5 µL	1pg-100ng

Table (3): Thermocycler program for One-Step quantitative RT-qPCR

Step	Temp. (°C)	Time	Cycle	Scaning
Reverse transcription	42 °C	10 min	Hold	
Enzyme activation	95 °C	3 min	Hold	
Denaturation	95.0 °C	15 sec	40	
Annealing	53.0 °C	15 sec		
Extension	72.0 °C	15 sec		

Quantitative reverse transcription PCR (qRT-PCR)

The gene expression was detected successfully by using new molecular technique which is Real time PCR (qRT-PCR) with used specific primer as described in table (1).

The data was studied according to Delta delta Ct (ΔΔCt) method, this method is the simplest one, as it is a direct comparison of Ct values between the target gene and the reference gene.

ΔCt (patients) = Ct (patients) mean – Ct (reference)mean

ΔCt (Controls) = Ct (controls) mean – Ct (reference)mean

ΔΔCt = ΔCt (patients) – ΔCt (controls)

Normalized target gene expression level = 2(-ΔΔCt)

Results and Discussion

Chemical compounds of the isolated essential oils

Essential oils were obtained by extraction of the *T. occidentalis* fresh leaves, thereafter; Gas chromatography and mass spectroscopy of the compounds were then analyzed. The chemical compositions of the EO are compiled in table (2), thirty-five compounds were identified from *T. occidentalis*. The abundant constituents of *T. occidentalis* were thujone (21.58%), camphor (18.92%) and Isobornyl acetate (9.71%).

Tsiri *et al*.(16) studied four *Thuja* essential oils species cultivated in Poland *T. occidentalis* "globosa", *T. occidentalis* "aurea", *T. plicata* and *T. plicata* "gracialis" the main constituents in all samples were the monoterpene ketones α- and β-thujone, fenchone and sabinene, as well as the diterpenesbeyerene and rimuene.

In a previous study(17) analyzed the composition of the essential oil from the aerial parts of *T. occidentalis* from. The 44 compounds identified in essential oil, α-Thunjone (cis) (50.14 %) had the highest values.

Table (4): Identified components of *T. occidentalis* Essential oil

Peak	*Thuja occidentalis*	R.Time	Area%	Mol. Weight
1.	Bicyclo[3.1.1]hept-2-ene	4.446	0.87	136
2.	alpha.-Pinene	4.672	1.52	136
3.	Camphene	5.091	7.50	136
4.	Thujene	5.589	8.57	136
5.	beta.-Myrcene	5.748	3.45	136
6.	Terpinolene	6.313	0.43	136
7.	Limonene	6.543	4.86	136
8.	Isopropyltoluene	6.821	3.01	134
9.	alpha.- Terpinol	7.190	1.52	136
10.	2,4,6-Trimethyl-1,3,6-heptatriene	7.725	0.27	136
11.	Fenchone	8.582	1.43	152
12.	Thujone	9.157	21.58	152
13.	Pentanone	9.192	1.02	152
14.	Terpineol	9.301	0.35	154
15.	Isothujol	9.366	0.29	154
16.	Dihydromyrcene	9.461	0.18	138
17.	Camphor	9.907	18.92	152
18.	alpha-Terpieol	10.158	0.58	154
19.	Isomyrcenyl acetate	10.203	1.43	196
20.	1-Methyl-4-(.alpha.-hydroxyisopropyl)benzene	10.393	0.21	150
21.	Carene	10.718	2.39	136
22.	3-Isopropenyl-2-methylenecyclohexyl acetate	10.898	0.27	196
23.	4,7-Dimethyl-1,6-octadien	11.115	0.25	154
24.	Solanone	11.298	0.30	194
25.	Isobornyl acetate	11.440	9.71	196
26.	alpha-Terpineol acetate	12.272	5.13	196
27.	alpha.-Limonene diepoxide	12.502	0.10	168
28.	2,6-Octadien-1-ol, 2,7-dimethyl	12.676	0.10	154
29.	alpha.-Bisabolene	12.956	0.19	204
30.	1,5,9,11-Tridecatetraene, 12-methyl	13.534	0.10	190
31.	Germacrene D	14.449	0.19	204
32.	Pentadecyn-1-ol	15.813	0.35	224
33.	Limonene oxide	16.218	0.08	152
34.	Aromadendrene oxide	19.217	1.23	220
35.	Isophyllocladen	19.669	1.63	272

Aspergillus flavus fungus

Five isolates were obtained from *A. flavus* fungus , from the University of Karbala and the University of Kufa, isolated from infected corn grain, Isolates were diagnosed.

Colonies appeared radially yellowish green and produced conidios structures in abundance and spherical or hemispherical conidia (Figure 1).

The results of the microscopic examination show that these isolates have a divided fungal spinning , from which transparent conidia carriers protrudes it tapered end with thick walls ranging in length from 900 - 1100 µm and its thickness of 10 - 13 µm completely covered in a row of structures, each carrying a series of spherical conidios. The diameter of each one reaches 40 µm (Figure 1). These characteristics corresponded exactly to the classification characteristics of fungi *A. flavus* of Markey *et al*. (18).

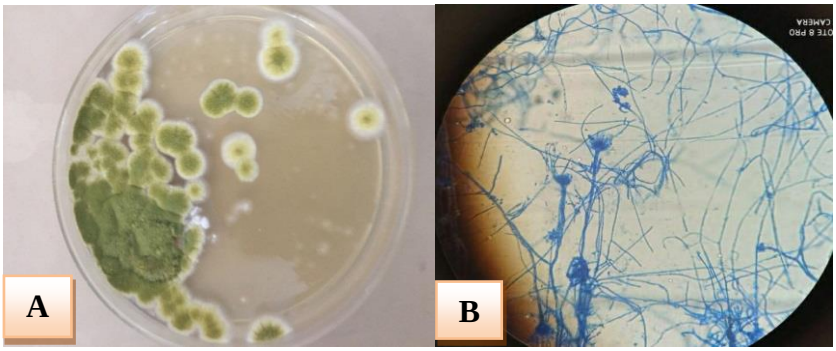

Figure (1): *Aspergillus flavus* colonies (A) on Sabouraud Dextrose Agar SDA medium (B) Under the microscope(50X)

Antifungal assay

Data presented in Figure (2), show the Inhibition percentage (IP%) of fungal growth when after treatment with Essential oil (*T. occidentalis*), antifungal (Nystatin), solvent (DMSO) and control, there significant differences ($P<0.05$) among different test material in their antifungal ability, where antifungals (Nystatin) with concentrate (MIC) 500 ppm excelled significantly by giving it the highest rate of inhibition 66.6%, However, the treatment with essential oil with concentrate (MIC) 500000 ppm gave good results in the inhibition of *A. flavus* fungus at a rate of inhibition 55.5%.

In fact, the antimicrobial activity of the entire extract could be due to their major or minor component alone or due to a synergetic effect between two or more components. Nevertheless, biological activities have been demonstrated to be linked to the most abundant components(19). The microbiological activity of the TEO may be due to its high content of thujone compound. This result agreed with Bellili *et al*. (20) who reported the high ability of the TEO in inhibiting the growth of *A.flavus* fungus. Whereas, the control treatment (*A.flavus* only) and treatment with DMSO solvent did not give any inhibition against *A. flavus*. This agrees with the findings of Togo *et al*.(21) who reported that the growth of *Aspergillus oryzae* fungus was not affected when treated with the DMSO solvent.

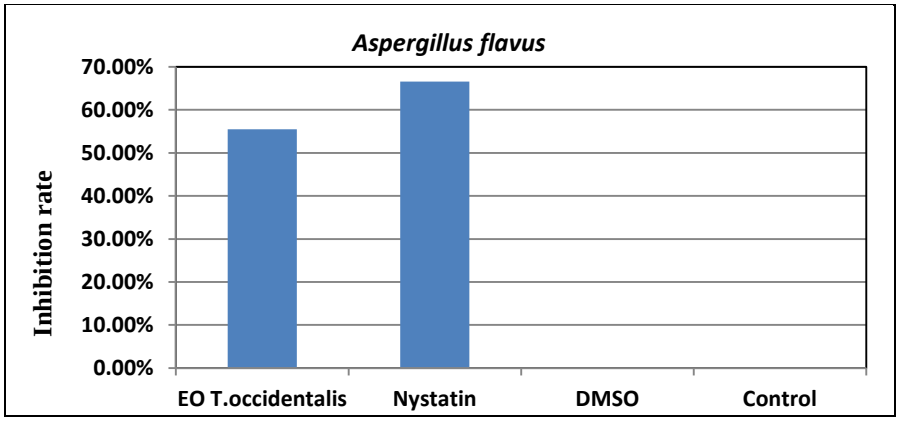

Figure (2): inhibition rate of *A.flavus* fungi after treatment test substance with a minimum inhibitory concentration (MIC).

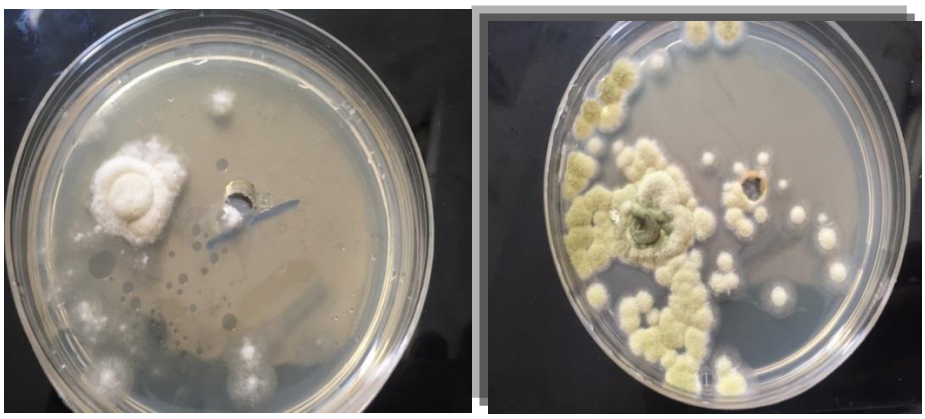

Figure (3): Identification minimum inhibition concentration (MIC) by Agar Well-Diffusion Method (ADM)

Molecular study

Gene Expression of aflR-2

The quality and purity of total RNA samples ranged between (1.7 to 1.9) ng/µl. This step in the present study content the estimation of aflR-2 gene expression in *A.flavus*, after treatment with TEO , antifungal (Nystatin) control , Solvent (DMSO) and nagative control fungi only (*A.flavus*). these treatments were chosen according to MIC values to each treatment. Its amplification was recorded as Ct value (cycle threshold). The house keeping gene used in the present study was β-tubulin gene for *A.flavus*. The purpose of using this genes in molecular studies is that its expression remains constant in the cells or tissues under investigation and different conditions.

The results after exposure of isolates to the essential oil antifungal compared to the control treatments, using a concentration the MIC dose (500000) ppm. a show significant decrease in gene expression . According to the results of a statistical analysis of the CT value, there were significant differences between treatments depending on LSD values at $P < 0.05$, the range of the value of Ct of the aflR-2 gene in this study was from (31.9 to 34.05). Whereas, the Ct value was for the reference gene (β-tubulin) (23.4 to 23.6) (Table5).

The results of RT-PCR showed that the expression of aflR-2 genes It did not differ significantly in the presence of TEO and Nystatin, But it decreased significantly compared to control samples and DMSO, Table (5) Figure (4). The reason for this is due to the components of the essential oil, which includes elements with a high biological activity such as (α-Thujone, β-Thujone, Fenchone, Camphene, Limonene, Terpineol, etc.) and this result is concordant with results(22).

What attracts our attention is that the treatment with essential oil result increased CT value, and this is a good indication of the low expression of the aflR-2 gene and thus a decrease in aflatoxin production of *A. flavus*.

Table (5): The Ct Value and ΔΔct of *aflR-2* Gene and Fold of Gene Expression.

Treatments	Ct value target gene	Ct value refrence gene	ΔΔct	Fold of Gene Expression
EO *T. occidentalis*	33.3	23.4	1.5	0.35
Nystatin	34.05	23.6	1.9	0.26
DMSO	31.98	23.4	0.13	0.91
Control	31.91	23.5	0	1
L.S.D				0.093
***P < 0.05**				

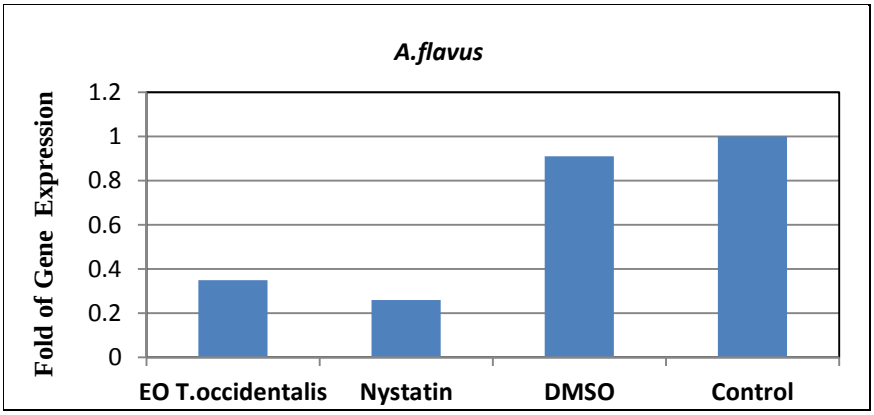

Figure (4): Fold of Gene Expression of aflR-2 Gene Depending on ΔΔCt Method.

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