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Dothideoxanthone, immunosuppressive and antioxidant agents from endophytic fungus *Aspergillus costaricensis*

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Abstract

The aim of the study was to identify endophytic fungi from different plant sources and which one produced a high level of xanthone compound and investigate that purified xanthone's immunosuppressant and antioxidant scavenger activity.

25 endophytic fungi were isolated from stems and leaves of healthy and mature plants of *Ficus carica*, *Capsicum frutescens*, and *Chenopodium mural* which were collected from Al-Qurna groves, Al-Zubair farm, and Safwan desert in Basrah province of Iraq respectively during December 2019 to February 2020. After that, fungal isolates were cultivated on semi-solid flour rice medium as fermentation medium for 40 days at 28oC to yield secondary metabolites. Then investigating crude extracts for each endophytic fungi by Gas Chromatography/MS, it is revealed that *Aspergillus costaricensis* (OM420244.1) extract from *F. carica* contains 9H-Xanthen-9-one,1-hydroxy-3,6-dimethoxy-8-methyl- with percentage 2.152% in retention time of 28.870 minutes. Purified xanthone by silica gel column and thin-layer chromatographed (TLC). Furthered characterized by LC-MS and MNR as Dothideoxanthone C16H14O5 (m/z 286.28). A significant positive correlation between Dothideoxanthone and immunosuppressant to mice splenic lymphocytes induced by LPS and Con A (IC50 14±0.49 and 11.73±0.14) respectively in comparing with cyclosporin A (IC50 7.67±0.07 and 6.91±0.08) as standard. Also, Dothideoxanthone had significant antioxidant scavenging activity in different tests (O-phenanthroline assay, Superoxide radical, and nitric oxide (NO) scavenging activity) with IC50 (14.90±0. 65, 7.73±0.04 and 171.31±13.93) respectively in comparing with standards ascorbic acid IC50 (54.02±7.69, >200 and 70.33±5.21) and BHT IC50 (108.84±7.16, >200 and >200) respectively.

The result reveals that dothideoxanthone purified from *A. costaricensis* secondary metabolites isolated from *F. carica* is found that it has moderately potent as immunosuppressant and highly potent as an antioxidant compound.

Keywords: endophytic fungi, xanthone, immunosuppressant, antioxidants

Introduction

Endophytic fungi are naturally living microorganisms inside plants' tissues and don't cause obvious pathological symptoms in the plant (Omomowo *et al*, 2023). Endophytes have been shown to produce a variety of bioactive metabolites in a single plant or microbe, making them a great novel source of medications for treating a variety of diseases and having potential uses in the food, cosmetics, agricultural, and medical industries (Fadiji and Babalola, 2020; Alfartosy *et al.*,2021). These secondary metabolites were divided into various classes according to functional groups, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, saponins, alkaloids, tannins, terpenoids, tetralones, xanthones, and many others (Gouda *et al.*, 2016). Endophytic fungi are capable of synthesizing novel bioactive chemical compounds with a variety of biological features, such as antiviral, antibacterial, antifungal, antiprotozoal, antiparasitic, antioxidant, immunosuppressive, and anti-cancer effects (Manganyi and Ateba, 2020).

Natural products serve important source in the drug discovery and medication development process. Many clinically applied drugs derived from or natural products (Newman and Cragg, 2020). Immunosuppressive agents are an important group of clinically used drugs for prevention organic transplant rejection and treatment of autoimmune diseases, such as rheumatoid arthritis, systemic lupus, multiple sclerosis, myasthenia gravis, and pemphigus (Dangroo *et al.*, 2016). Despite the great benefits, these immunosuppressive medications have certain unavoidable serious adverse effects, such as kidney and liver damage, infection, cancer, and others (Smith *et al*, 2003). Therefore, it is important to develop novel, safe, and effective immunosuppressive agents (Ye *et al*., 2022).

An antioxidant agent is an important compound that could prevent damage to cells caused by free radical molecules during oxidative stress (Nuraini *et al*., 2019). All cells regularly produce free radicals as part of the normal metabolic process of reactive oxygen species (Ibrahim *et al*., 2021). Excessive accumulation and production of ROS may result in oxidative damage, mutation, cellular toxicity and cell death due to the structural change of cellular molecules (Gautam *et al*., 2022). Different pathogenesis of diseases had been significantly related to oxidative stress such as aging, malignancies, Alzheimer's disease, autism, cardiovascular disease, diabetes, cataracts, inflammatory disease and rheumatoid arthritis (Lü *et al*., 2010 and Rasheed *et al*., 2019).

Xanthones are a class of heterocyclic natural secondary metabolites from plants, fungi, and lichen offer greater structural diversity and pharmacological value. Thus, they are attractive to researchers because of wide bioactivities as anti-neoplasm, anti-diabetes, hepatoprotective, antihyperlipidemic, antiatherogenic, anti-inflammatory, antiulcer, analgesic, anti-bacterial, antifungal, anti-leprosy, antimalarial neuroprotective, anti-HIV (Negi *et al*.,2013; and, Soares *et al*., 2022). Furthermore, xanthones had been reported as potent immunosuppressants (Song *et al*., 2013; and, Liu *et al*., 2016) and pronounced potential as antioxidants by reacting with the free

radicals either through a single electron transfer or hydrogen atom transfer mechanism (Salman *et al*., 2019).

Materials and Methods

Plants collection

Fresh leaves and stem samples were collected from healthy fig trees *F. carica* from Al-Qurna groves, *C. frutescens* from one of the farms in the Al-Zubair region, and *C. mural* from Safwan desert region in Basrah governorate of Iraq. Each sample was kept separately in a sealed sterile labeled plastic bag, then transferred to the clinical and physiological laboratory research of fungi in the Department of Biology, College of Sciences, University of Basrah, and stored at 4ºC in the fridge until further studies, while, the plant samples were classified by the staff of plant taxonomy laboratory.

Isolation of endophytic fungi

The isolation of entophytic fungi from plant samples was carried out according to (Rimbawan *et al*.,2018) as follows: The dust and derbies were removed from all collected plant samples by washing them under running tap water and dry at room temperature, then divided samples to smaller parts (2-3 cm size), after that, the segments of the plant were sterilized by immersion them for two minutes in sodium hypochlorite 5.25% solution, then washed again with sterile distal water, followed by immersion for two minutes in 70% ethanol, finally, another rinse with sterile distal water was done for all parts of plants. Sterilized segments cutting into smaller parts (1x1 cm) by using a sterile blade and placed (3-4 parts) on Petri dishes containing Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) with a duplicate for each medium and incubated for (5–14) days at 28°C. To acquire a single isolate of endophytic fungi, the fungal colonies of endophytic fungi were purified by transferring a little piece of each colony onto a different PDA or SDA medium and cultivating under the same conditions as previously.

Identification of Endophytic Fungi

Morphological identification was carried out by examining both macroscopic and microscopic properties. Furthermore, the final identification was achieved by molecular approach (Degruyter *et al*.,2013; Spatafora *et al*., 2016; Wanasinghe *et al*., 2017).

The genomic fungal DNA was extracted using a Presto TM Mini gDNA Yeast kit, (Geneaid, New Taipei City, Taiwan). Appropriate amount of activated fungal colonies on PDA were collected for extraction process, whilst, the region of 28S large subunit of rDNA was utilized for amplification step that applied by the general primers Null1(NL1) F-5'- GCA TAT CAA TAA GCG GAG GAA AAG -3' and Null4 (NL4) R-5'- GGT CCG TGT TTC AAG ACG G -3 under the following conditions: 94°C for 5 min, then 30 cycles of 94°C for 30 sec, 56°C for 45 sec, 72°C for 1 min, and final extension at 72°C for 5 min, however, the total volume of PCR composition was 25 µl consisted of 12.5 µl Master mix (Bioneer, Korea), 1.5 µl of individual primer, 1.0 µl of fungal DNA, and 9 µl of nuclease free water (Mirhendi *et al*. 2008).

Sequencing

PCR products of fungal isolates were sent to Macrogen company (Seoul, South Korea) for sequencing, then after, the sequences were treated by Chromas 2.6.6 and deposited in Basic Local Alignment Search Tool (BLAST) in comparable with reference isolates of National Center of Biotechnology Information (NCBI)and compared with those available in Gen Bank via BLAST searches.

Fermentation and Extraction

The fungal isolated was activated on PDA at 28 °C for 7 days. Five blocks of 6 mm of diameter of activated isolates were transverse to Erlenmeyer flasks (250 ml) contained autoclaved semisolid flour rice fermentation media, which it prepared weighting 12.5 gm of flour rice that added to 3‰ of saline water (3gm of NaCl in 1000 ml of distilled water). The media was incubated for 40 days at 28°C. After the incubation period, the fungal growth and flour rice substrate was extracted by adding EtOAc (250 ml), repeated this process three times. After that, the recovered organic solvent was allowed to totally evaporate at room temperature.

Purification of compounds

Eluted silica gel column with toluene-methanol (95:5) was loaded with dissolved extract in toluene-methanol (95:5). Different color-related fractions were collected together and dried. To isolate pure chemicals, fractions eluted from the column were thin-layer chromatographed (TLC) on silica gel 60 F254-coated plates (thickness 0.25 mm) eluted with toluene-methanol.

Quantitative analysis of liquid chromatography-mass spectrometry (LC-MS/MS) profiling

The compounds were sent to the Republic of Iran / Kashan University / Central Laboratory for (LC-MS/MS) investigation to confirmation of molecular weight and structure of purified compound.

Fourier Transform Infrared Spectroscopy (FTIR)

The infrared spectrum of the purified compound by using the FT-Infra-Red Spectrum device at wavelengths 400-3800 nm at the Polymer Research Center / the University of Basrah.

Nuclear Magnetic Resonance (NMR) Spectroscopy

purified compound was characterized by using 1H, 13C, and 2D NMR spectroscopy. 1H NMR (CDCl3, 400 MHz) = 1.67 (s, 15H, C5Me5), 2.38 (s, 3H, Me), and 13C1H NMR (CDCl3, 100 MHz) = 9.2 (C5Me5), 21.4 (Me), and 95.5 (d).

Immunosuppressant Activity

Preparation of lymphocytes from the mouse spleen

The BALB/c male mice weight 20±2 g and between 6 and 8 weeks old were provided by the College of Veterinary Medicine/ University of Basrah. By cervical dislocation, BALB/c mice were sacrificed, and their spleens were harvested and kept in RPMI 1640 before being minced with surgical shears and put through a cell strainer to create a single-cell suspension. Ammonium

chloride was used to lyse the erythrocytes (0.75 %, w/v). The resultant cells were then washed three times with PBS (pH = 7.4) and resuspended in full RPMI 1640 after centrifugation (1000 rpm at 4°C for 5 min). Trypan blue dye exclusion was used to count the cells using a hemocytometer, and the cell viability was above 95%.

The experiment involving lymphocyte proliferation employed the acquired cells. (Li *et al*., 2010).

Antiproliferation immune cells

The immunosuppressive effect of the purified compound in vitro was measured by using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Cyclosporin A was used as positive control. Splenic lymphocytes density was 2×10^6 cells were cultured in a plate 96- well. LPS and Con A stock solution was injected into each well respectively (the final concentration: 5 mg/mL). Then five different concentrations were added to each well from the purified compound and cyclosporin A (3.125,6.25, 12.5, 25, 50,100 µg/mL) respectively. The plates were incubated for 48h at 37°C, with a 5% CO2 atmosphere, then 20 mL MTT reagent was injected for each well 4 h before the end of the incubation. When the incubation was finished, the plate was centrifugated and the supernatants were removed, after that 100 mL of DMSO was injected into each well. The absorbance was measured by a microplate reader at a wavelength of 570 nm (OD570 nm). Every concentration was performed in triplicate, and every assay was measured three times to ensure accuracy. The average inhibition ratio and the IC50 value were determined by assisting the appropriate software program (Graph Pad Prism Version 8.0.2 (263)) (Song *et al*., 2013).

Antioxidant Activity

O-phenanthroline assay

By measuring the absorbance of the combination of different concentrations of 2 ml (3.125, 6.25, 12.5, 25, 50, 100, and 200 µg /ml) of purified compound and ascorbic acid as standard with reaction mixture composed from 1 ml O-phenanthroline (0.005gm in 10 ml methanol), 2 ml ferric chloride 200 M (3.24 mg in 100 ml DW). After that kept for 10 minutes at room temperature. The percentage of inhibition was calculated using the following formula after the combination's absorbance was measured at 510 nm(Re *et al*., 1999).

Inhibition % = $\frac{A_{contl-A_{sample}}}{A_{control}} \times 100$

Superoxide radical scavenging activity by DMSO alkaline method

The investigation was done by adding 0.1 ml of Nitro-blue tetrazolium (NBT) (1 mg/ml) to a reaction mixture containing 1 ml of alkaline DMSO (containing 1 ml DMSO and 0.1 ml of 5 mM NaOH dissolved in water) and 0.3 ml of the purified compound and standard of ascorbic acid and BHT (3.125,6.25,12.5, 25, 50, 100, and 200 µg /ml) in freshly distilled DMSO at varying concentrations.

The absorbance at 560 nm was measured, and the % inhibition was computed using the following method. (Srinivasan *et al*., 2007).

Inhibition % = $\frac{A_{contl-A_{sample}}}{A_{control}} \times 100$

Determination of nitric oxide (NO) scavenging activity.

Different concentrations of each of the purified compound (3.125, 6.25, 12.5, 25, 50, 100, and 200 µg /ml) were dissolved in DMSO were combined with sodium nitroprusside (10 mM) in PBS and incubated for 150 minutes at room temperature. The control was the same reaction mixture with an equal quantity of DMSO but no chemicals. 0.5 ml of Griess reagent (containing sulfanilamide, H3PO4, and N-(1-naphthyl) ethylenediamine dihydrochloride at concentrations 1%, 2%, and 0.1% respectively) was added after the incubation time.

At 546 nm, the absorbance of the chromophore generated in the reaction was then measured. Ascorbic acid and BHT were employed as positive controls (Kalala, *et al*., 2015)**,** and the percentage of inhibition was computed using the following formula:

$$\text{Inhibition \%} = \frac{A_{conl-A_{sample}}}{A_{control}} \times 100$$

Statically analysis

The static analysis was employed by using SPSS Statistical Packages of Social Sciences (Version 26; USA) to determine means ± SD for all data. Also, the least significant difference test (LSD) was used to test the difference between means; Statistical significance was considered significant at $p < 0.05$.

Resulte

Isolation and identification of endophytic fungi

25 endophytic fungi were isolated from the stems and leaves of healthy and mature plants of *Ficus carica, Capsicum frutescens, and Chenopodium mural*. All isolates were arranged with 7 genera and 10 species. Isolated fungi were included (*Alternaria alternata, Aspergillus costaricensis, A. flavus, A. fumigatus, A. niger, Cladosporium perangustum, Penicillium chrysogenum, Rhodotorula mucilaginosa, Stemphylium eturmiunum, Talaromyces atroroseus*).

Molecular identification of endophytic fungi

The results of the molecular diagnosis showed that the 25 fungal isolates sent to Macrogen were all diagnosed using the NL primer, with high similarity rates, as shown in table (1).

Species-level identification of fungi may be challenging due to the tremendous variety of their ecology and anatomy; therefore, it looks difficult to design an accurate species identification method applicable to all fungi. Today, DNA sequences from diverse places have grown into potent molecular data for quick identification and surpassing the numerous sets of traditional criteria used to characterize fungal species. Furthermore, Choosing the possible DNA barcode region(s) is essential for identifying biological data and attributing them to a certain species (Tekpinar and Kalmer, 2019; Taufiq and Darah, 2018). Studies underline the need of undertaking molecular diagnostics owing to its significance in precisely identifying species and genera (Tedersoo and Nilsson, 2016; Bálint *et al*., 2016).

Table (1): Fungal isolates registered in the gen bank with their accession number.

N	Diagnostics using NL1- NL4	Length	Identical to strain	Accession no.	Source
1	*Alternaria alternata*	584 bp	100%	OM420234.1	*Ficus carica*
2	*A. alternata*	587 bp	100%	OM420235.1	*F. carica*
3	*A. alternata*	595 bp	100%	OM420236.1	*F. carica*
4	*A. alternata*	570 bp	99.65%	OM420237.1	*Capsicum frutescens*
5	*A. alternata*	568 bp	99.82%	OM420238.1	*F. carica*
6	*A. alternata*	580 bp	99.31%	OM420239.1	*F. carica*
7	*A. alternata*	585 bp	100%	OM420240.1	*C. frutescens*
8	*A. alternata*	592 bp	100%	OM420241.1	*C. frutescens*
9	*A. alternata*	578 bp	100%	OM420242.1	*Chenopodium mural*
10	*A. alternata*	584 bp	100%	OM420243.1	*C. murale*
11	*Aspergillus costaricensis*	584 bp	99.66%	OM420244.1	*F. carica*
12	*A. costaricensis*	586 bp	99.83%	OM420245.1	*C. frutescens*
13	*Aspergillus flavus*	590 bp	100%	OM420252.1	*F. carica*
14	*Aspergillus fumigatus*	587 bp	98.12%	OM420257.1	*F. carica*
15	*Aspergillus niger*	574 bp	99.83%	OM420276.1	*C. frutescens*
16	*A. niger*	588 bp	100%	OM420277.1	*C. murale*
17	*A. niger*	584 bp	99.66%	OM420278.1	*C. murale*
18	*Cladosporium perangustum*	554 bp	99.82%	OM420285.1	*C. frutescens*
19	*P. chrysogenum*	589 bp	100%	OM420490.1	*C. frutescens*
20	*P. chrysogenum*	580 bp	100%	OM420491.1	*C. murale*
21	*P. chrysogenum*	584 bp	99,83%	OM420492.1	*C. murale*
22	*Rhodotorula mucilaginosa*	578 bp	100%	OM420494.1	*F. carica*
23	*R. mucilaginosa*	583 bp	100%	OM420495.1	*F. carica*
24	*Stemphylium eturmiunum*	553 bp	100%	OM420553.1	*F. carica*
25	*Talaromyces atroroseus*	556 bp	99.64%	OM420714.1	*F. carica*

Aspergillus species have been identified as endophytes, parasites, saprophytes, and human pathogens, and had the capacity for producing metabolites with diverse chemical compositions and bioactive components (Zhang *et al*., 2018). Xanthones are one of a compohat presented in the bioactive raw material of *A. costaricensis* which is isolated from *F. carica* and this finding agrees with the studies of (Frisvad *et al.,* 2008 and Almanaa *et al.*, 2022) who showed that isolation the same chemical bioproducts.

Characterization of pure Compound Dothideoxanthone

This compound was found to be the known Dothideoxanthone, previously isolated for the first time from the broth extract of the Dothideomycetes CMU-99 (Boonyaketgoson *et al*., 2015)
The LC-MS of this compound gave a $[M+Na]^+$ ion peak at *m/z* 309.03, calculated for $[M+Na]^+$ 309.03 which indicated a molecular formula $C_{16}H_{14}O_5$ (*m/z* 286.28). A double bond equivalence of ten was calculated for this compound figure (3). The FTIR spectrum showed absorptions bands at 3417 cm^{-1} for O-H stretch, 2923 and 2853 cm^{-1} for C-H stretch 1731 cm^{-1} and 1651 cm^{-1} for C=O stretch in the ing, 1616 cm^{-1} for C=C aromatic stretching bands, 1159 cm^{-1} for C-O stretch figure (4).
The ^{13}C NMR spectrum, displayed sixteen carbon resonances, including nine fully substituted carbons, four methine, and three methyl group resonances including two methoxy groups, typical of a xanthone structure (table 3) and Figure (2 and 7).

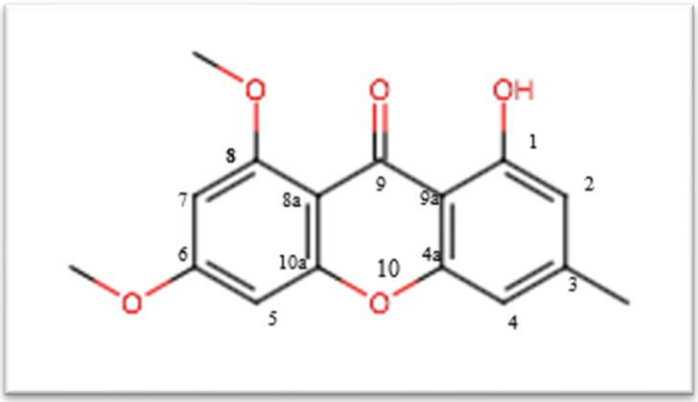


Figure (2): Structure of compound Dothideoxanthone

The ^1H NMR spectrum showed a proton resonance at δ_H 2.34 ppm integrated for three protons which were seen to correspond to the carbon resonance at δ_C 20.69 ppm in the HSQC spectrum. This resonance was assigned to C-3 as it showed a correlation in the HMBC spectrum with the resonance at δ_c 107.37 (C-2) and 1δ_c 67.39 (C-3) ppm. The two methoxy groups resonance at δ_C 55.42 and 56.11 that corresponded to two singlets protons resonances at δ_H 3.90 (3H) and 3.97 (3H) ppm in the HMBC spectrum were sig on and oms respectively due to its correlations with carbon resonance at δ_c 162.67 (C-8) and 160.67 (C-8) ppm respectively in the HMBC (figure 5, 6, 8 and 9)
The two singlet aromatic proton resonances at δ_H 5.97 and 6.94 s assigned to C-2 and C-4 respectively due to its correlation with fully substituted carbon resonances at δ_c 167.39 (C-3), 104.4 (C-9a), 153.33 (C-4a) and methin group resonance at δ_c 107.37 (C-2) in the HMBC spectrum.
The two methine aromatic carbon resonances at δ_c 97.26 and 97.83 which corresponded to doublet aromatic proton resonances at δ_H 6.37 and 6.56 with coupling constant *J*=2.0 Hz were assigned to be C-7 and C-5. These resonances showed correlations, seen in the HMBC spectrum, with C-7 (δ_c 97.2 6), C-5 (δ_c 97.83), and C-8a (δ_c 108.45).
A comparison of the spectroscopic data for this compound with that found in the literature (Boonyaketgoson *et al*.,2015) confirmed that the compound was Dothideoxanthone.

Table (3): NMR data for compound Dothideoxanthone in Chloroform

N.	^{13}C NMR (125 MHz)	^{1}H NMR (Chloroform, 500 MHz)	^{1}H NMR (Acetone-d6, 400 MHz) Ref1	HMBC
1	161.54 C			
2	107.37 CH	5.97 br s	5.91 br s	2, 3
3	167.39 C			
4	101.05 CH	6.94 br s	6.48 br s	2, 9a, 4a
5	97.83 CH	6.56 d *J*=2.0 Hz	7.05 (d, J = 2.4 Hz	7, 8a
6	160.67 C			
7	97.26 CH	6.37 d *J*=2.0 Hz	6.93 (d, J = 2.4 Hz	5, 8a
8	162.67 C			
9	184.24 C			
10a	141.11 C			
8a	108.45 C			
9a	104.40 C			
4a	153.33 C			
1-OH	-	14.97	11.94	
8-O$\underline{C}H_3$	55.42 CH_3	3.90 s	3.74 s	8
6-O$\underline{C}H_3$	56.11 CH_3	3.97 s	3.81 s	6
3-$\underline{C}H_3$	20.69 CH_3	2.34 s	2.16 s	3-$\underline{C}H_3$, 2, 3

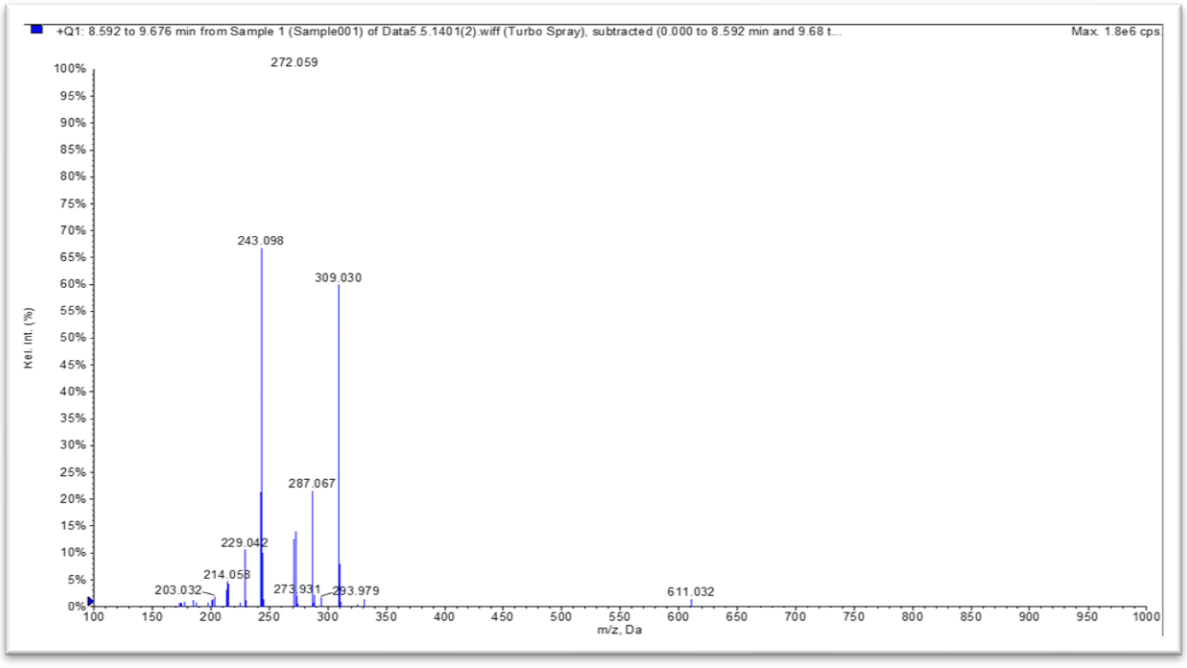


Figure (3): Mass spectrum for compound Dothideoxanthone

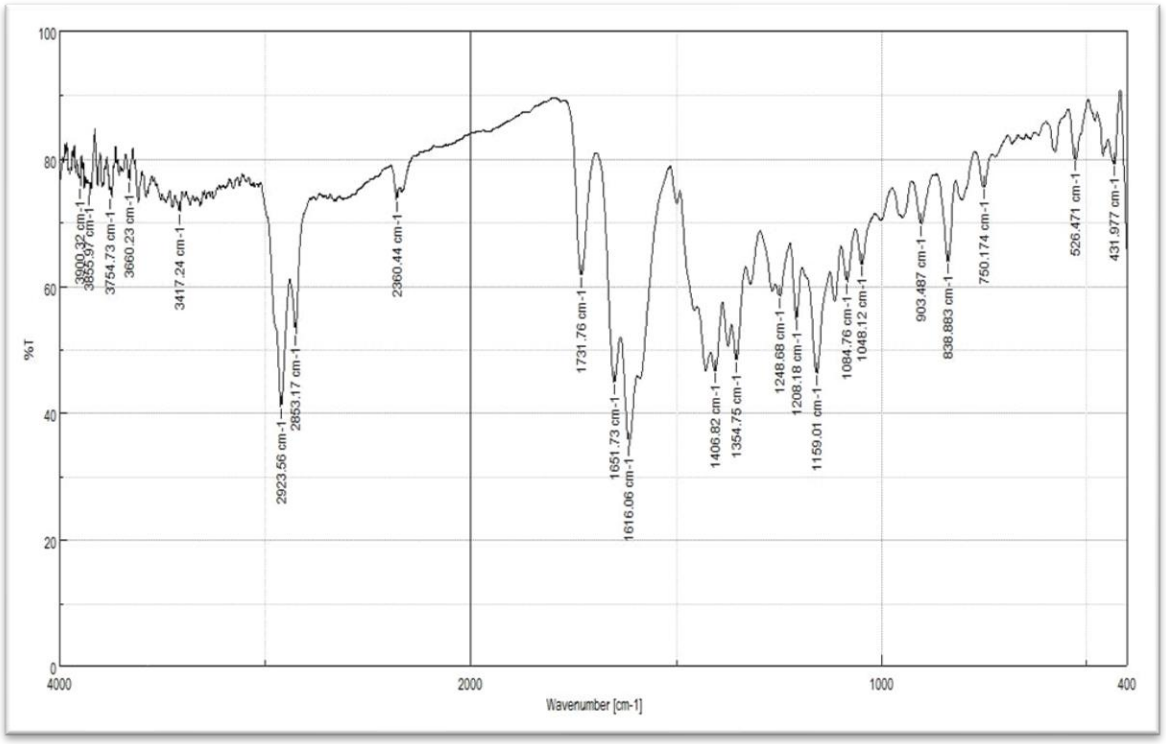


Figure (4): FTIR spectrum for compound Dothideoxanthone

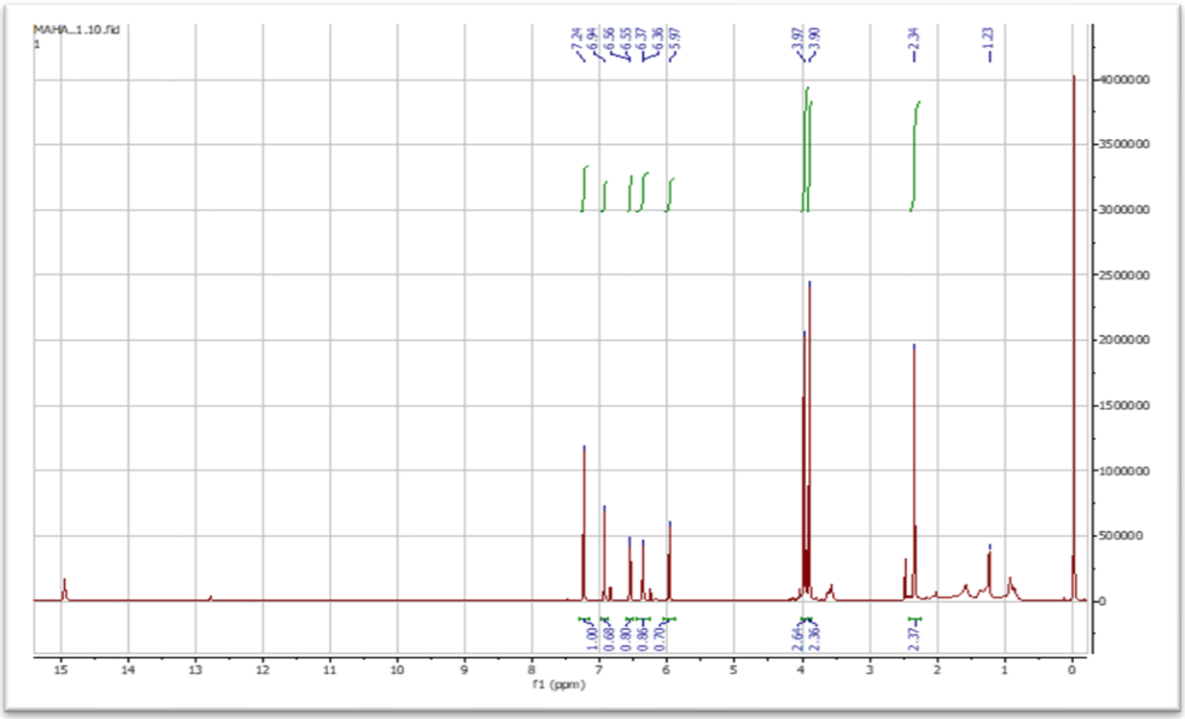


Figure (5): ^{1}H NMR spectrum for compound Dothideoxanthone in $CDCl_3$

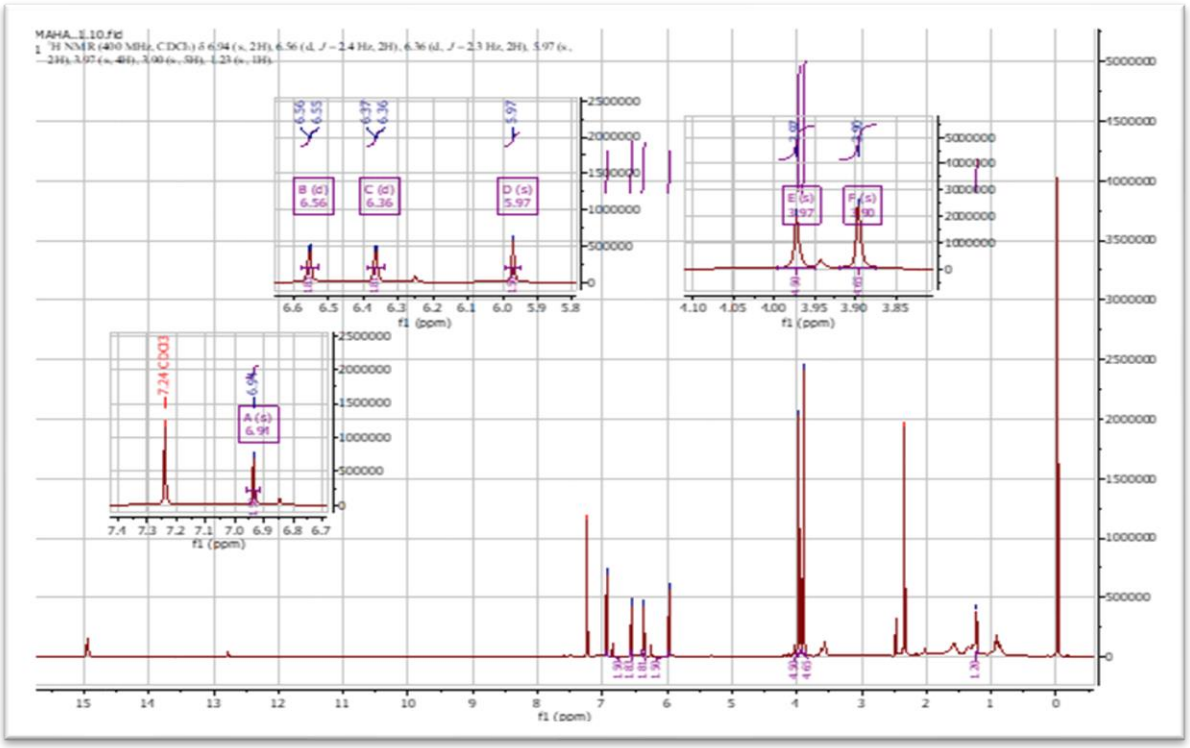
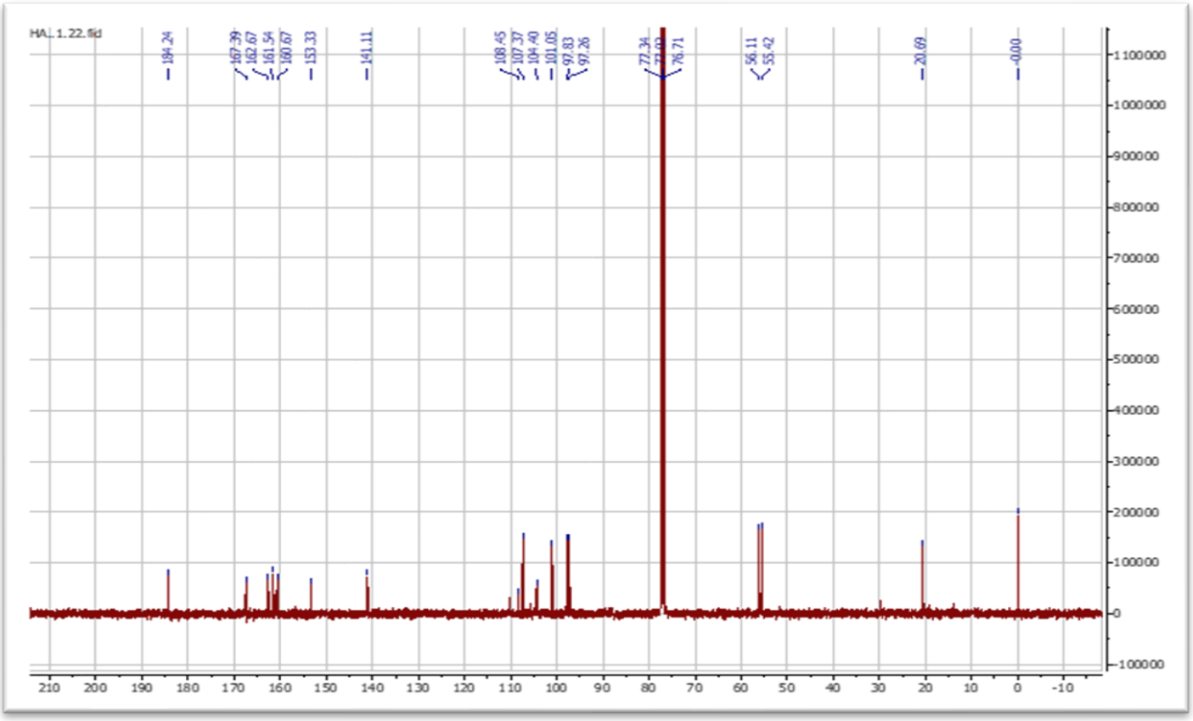


Figure (6): ^{1}H NMR spectrum for compound Dothideoxanthone in CDCl



Figure(7): ^{13}C NMR spectrum for compound Dothideoxanthone in $CDCl_3$

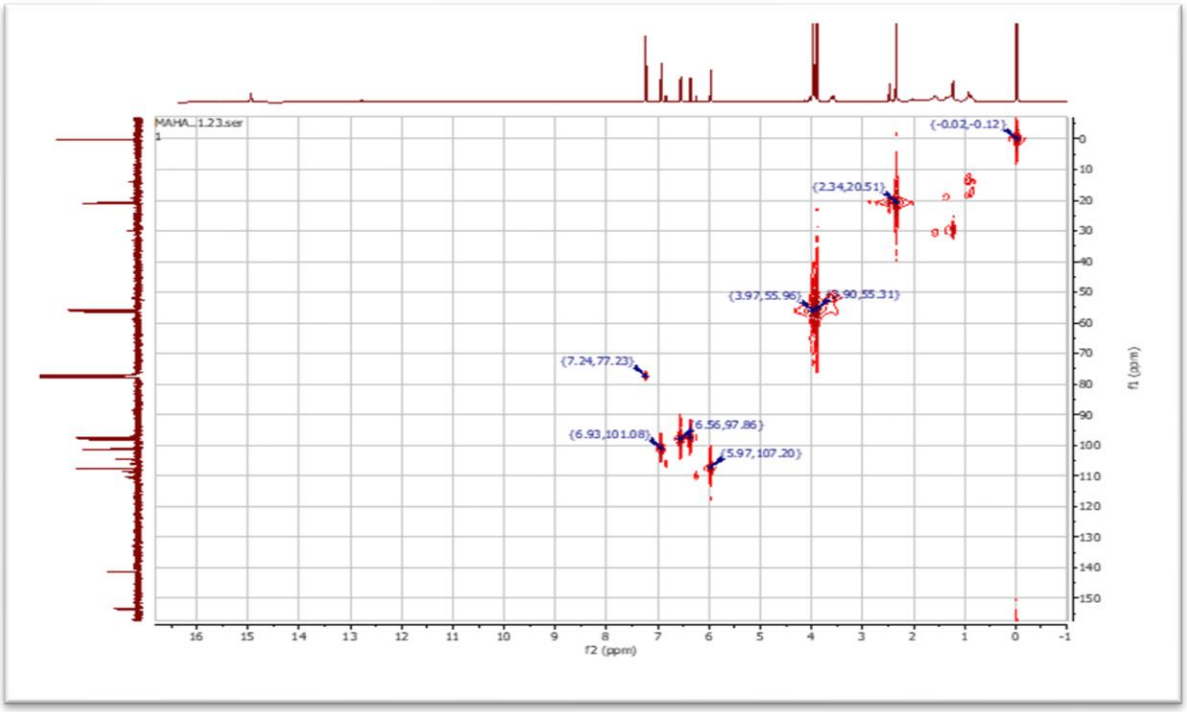


Figure (8): HSQC, DEPT spectrum for compound Dothideoxanthone in $CDCl_3$

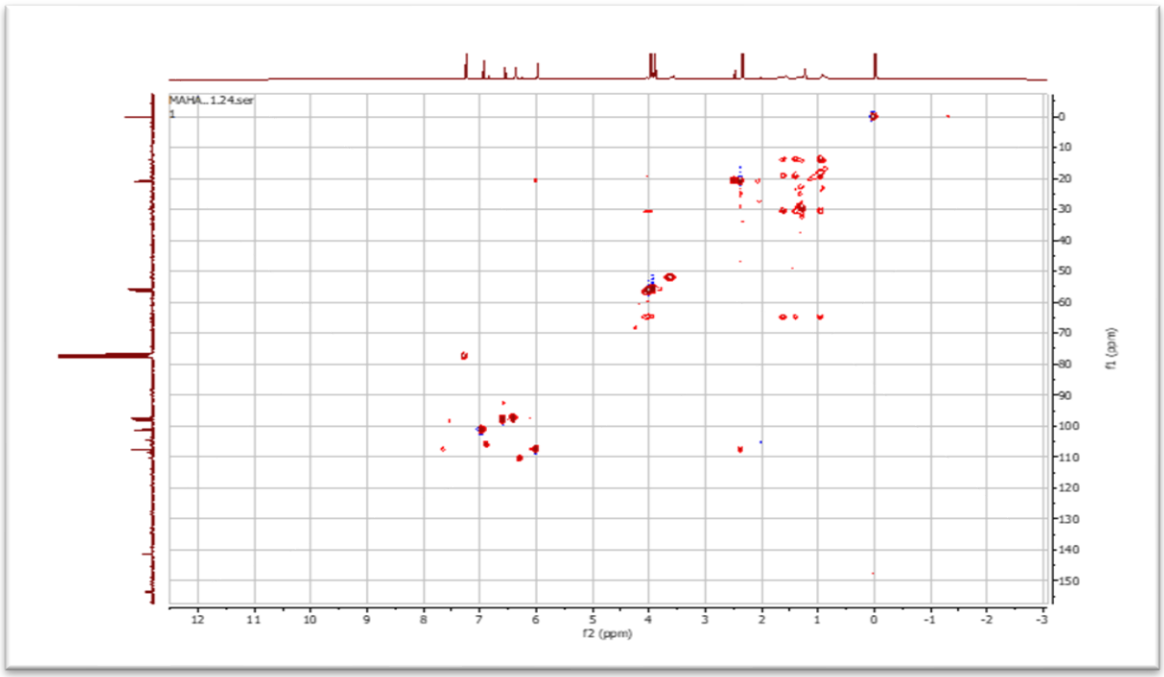


Figure (9): HMBC spectrum for compound Dothideoxanthone in $CDCl_3$

Antiproliferation activity Dothideoxanthon

The immunosuppressant activity of Dothideoxanthone was evaluated on mouse splenic lymphocytes induced by LPS (B-cells) and ConA (T-cells) in vitro while lymphocytes inhibition

was measured by MTT-assay. The inhibitory effect of Dothideoxanthone on lymphocytes proliferations (induce by LPS and ConA) was increased by multiplication their concentrations as in table (4 and 5), Dothideoxanthone had been shown moderate inhibitory comparison with cyclosporin A.

Table (4): *In vitro*. Inhibitory effect of Dothideoxanthone and Cyclosporin A on lymphocyte proliferation induced by LPS

Concentration µg/ml	Inhibition of Lipopolysaccharide -induced lymphocyte proliferation				
	Optical density			%Inhibition rate	
	Dothideoxanthone	Cyclosporin A	Total	Dothideoxanthone	Cyclosporin A
3.125	0.3±0.001	0.23±0.001	0.28±0.04	29.31	46.01
6.25	0.25±0.004	0.21±0.002	0.26±0.03	39.14	50.4
12.5	0.24±0.004	0.18±0.001	0.23±0.03	43.69	56.87
25	0.22±0.002	0.16±0.002	0.21±0.04	47.68	62.86
50	0.199±0.001	0.13±0.002	0.2±0.04	50.88	67.89
100	0.17±0.003	0.12±0.001	0.18±0.04	53.75	71.49
Total	0.23±0.04	0.17±0.04	0.23±0.05		
IC50 µg/ml	-	-	-	14±0.49	7.67±0.07

Table (5): *In vitro*. Inhibitory effect of Dothideoxanthone and Cyclosporin A on lymphocyte proliferation induced by ConA

Concentration µg/ml	Inhibition of ConA -induced proliferation lymphocyte				
	Optical density			%Inhibition rate	
	Dothideoxanthone	Cyclosporin A	Total	Dothideoxanthone	Cyclosporin A
3.125	0.28±0.001	0.21±0.001	0.27±0.04	33.56	50.69
6.25	0.26±0.002	0.196±0.002	0.25±0.03	40.74	40.74
12.5	0.23±0.003	0.18±0.003	0.22±0.03	47.92	59.26
25	0.21±0.002	0.15±0.001	0.2±0.03	52.55	66.89
50	0.19±0.004	0.13±0.002	0.18±0.03	55.48	71.06
100	0.16±0.001	0.11±0.003	0.16±0.03	62.19	73.61
Total	0.22±0.04	0.1612±0.04	0.21±0.05	-	-
IC50 µg/ml	-	-	-	11.73±0.14	6.91±0.08

The success of organ transplantation operations is related to the administration of immunosuppressive medications during the period of treatment. Also, the current immunosuppressant drugs have good therapeutic results still associated with serious side effects including kidney toxicity and neurogenic toxicity, increase risks of infection, neoplasm, post-transplant diabetes mellitus, dyslipidemia, and hypertension. In order that, agreed attention to find and discover new immunosuppressive agents to be more effective with less or no side effects (García-Carrasco *et al*., 2009.; van Gelder et al., 2014). The mechanism of xanthones' function as an immunosuppressive agent was not entirely known, some others believed that they act by inhibiting immune cells and inflammatory mediators (Khattab and Farag, 2021). Additionally, Gunter *et al*., (2020) suggested that xanthone may suppress the activation and multiplication of T-lymphocytes, an immunological cell type that plays a major role in the immune response. Additionally, xanthones may decrease the generation of pro-inflammatory cytokines, which are inflammatory signaling molecules. In the present study, Dothideoxanthone showed a different inhibitory effect on lymphocytes with moderate potency in comparison with cyclosporin A, and these results coincided with previous studies that reported the immunosuppressive activity of xanthones (Fujimoto *et al*., 2006.; Song *et al*., 2013.; and Liu *et al*., 2016).

Antioxidant Activity

The free radical scavenging activity (FRSA) of Dothideoxanthone in comparison with ascorbic acid and BHT equivalents as standers were estimated. The activity was determined in vitro by the flowing methods: O-phenanthroline, Superoxide radical scavenging activity by DMSO alkaline, and determination of nitric oxide (NO) scavenging activity.

O-phenanthroline assay

O-phenanthroline is a colorimetric iron-detecting reagent and, the assay included the reduction of Fe^{3}+ to Fe^{2}+ ion by an antioxidant. After forming Fe2+ion, o-phenanthroline interacted with it to produce a crimson orange complex (Yefrida *et al*., 2018). From the results table (6), Dothideoxanthone had the ability to reduce Fe3+ and increase reduction with increasing its concentration. Dothideoxanthone had strong antioxidant scavenger activity (IC50 = 14.90±0. 65) compared with ascorbic acid (74.46±1.19) and BHT (64.5±3.12) standards.

Table (6): O-phenanthroline radical scavenging activity of Dothideoxanthone and standards the Ascorbic acid and BHT.

concentration µg/ml	% Inhibition in O-phenanthroline assay			
	Dothideoxanthone	Ascorbic acid	BHT	Total
3.125	25.52±1.46	7.48±0.78	2.51±0.45	10.76±8.16
6.25	36.72±2.9	10.81±0.77	10.77±1.31	18.2±10.11
12.5	47.61±1.9	21.75±2.07	20.33±2.51	28.65±10.35
25	58.5±0.82	38.18±1.3	27.85±2.16	38.65±11.24
50	66.6±1.78	49.84±2.02	38.46±2.07	49.53±10.75
100	75.72±1.75	60.69±2.81	48.42±1.16	60.07±10.75
200	87.31±2.78	74.46±1.19	64.5±3.12	72.93±10.11
Total	56.85±20.76	37.6±24.28	30.4±20.65	39.83±23.18

IC_{50} µg/ml	14.90±0. 65	54.02±7.69	108.84±7.16	-

Superoxide radical scavenging activity by the DMSO alkaline method

The principle of this assay depends on conversation nitroblue tetrazolium into a formazan dye that can be detected at 560nm at room temperature, this colorimetric change was formed by superoxide action that is considered a stable solution produced by adding sodium hydroxide to DMSO saturated with air (Srinivasan *et al*., 2007). The antioxidant ability of Dothideoxanthone showed inhibition action of NBT reduction by superoxide at all concentrations in a table (7), hence the maximum inhibition was seen at the highest concentration (200 µg/ml) for Dothideoxanthone. Besides highly potent superoxide scavenging activity (IC50 = 7.73±0.04) for Dothideoxanthone in spite of the values standards (IC50 >200 µg/mL), with a significant difference at p (0.05).

Table (7): Superoxide radical scavenging assay by alkaline DMSO of Dothideoxanthone and standards the Ascorbic acid and BHT

Concentration µg/ml	% Inhibition in DMSO alkaline method			
	Dothideoxanthone	Ascorbic acid	BHT	Total
3.125	55.4±1.39	10.3±0.78	7.42±0.51	34.44±21.88
6.25	59.42±1.03	13.7±3.57	11.57±0.88	38.74±22.32
12.5	63.643.24	19.281.15	14.38±0.52	41.43±21.26
25	69.41±3.2	29.59±1.01	16.83±2.96	47.62±21.71
50	75.24±1.81	36.35±1.13	18.58±1.02	52.5±22.57
100	85.52±1.67	43.38±1.83	23.02±0.18	58.51±23.49
200	95.82±1.93	49.51±1.76	29.12±2.07	64.89±26.43
Total	72.06±13.93	28.87±14.36	17.28±5.83	48.45±24.45
IC_{50} µg/ml	7.73±0.04	>200	>200	-

Determination of nitric oxide (NO) scavenging activity.

NO is a vital component in the control of physiological activities such as blood pressure, immunological response, and brain activity under normal physiological settings. However, excessive NO generation may result in tissue damage and is linked to inflammatory disorders such as atherosclerosis and hypertension (Pacher et al., 2007). In order to treat chronic inflammatory disorders, researchers have focused increasingly on identifying natural antioxidants that may serve as powerful regulators of NO generation (Rudrapal *et al*., 2022). Conforti *et al*., (2011) recorded an inverse association between the intake of foods rich in antioxidants and the lowering of risk factors for certain human illnesses.

NaNO2-free radicals are created during the decomposition of sodium nitroprusside in solution at a physiological pH, whereas Nitrites are created when NO radicals react with oxygen in an aerobic environment. In this investigation, nitric oxide was produced from sodium, and nitroprusside was quantified using a modified Griess reagent (Udenigwe *et al*., 2009).

Dothideoxanthone had been revealed to suppress effect production NO by increasing its concentration table (8) and IC50 (171.31±13.93) was a good scavenging factor of nitrite radical in comparison with ascorbic acid (70.33±5.21) and BHT (IC50>200).

Table (8): Nitric oxide activity of Dothideoxanthone and standards the Ascorbic acid and BHT

concentration µg/ml	% Inhibition in NO scavenging activity assay			
	Dothideoxanthone	Ascorbic acid	BHT	Total
3.125	7.79±0.46	15.5±1.14	2.95±0.46	5.84±5.57
6.25	17.5±1.09	19.06±1.29	5.96±0.41	11.15±6.27
12.5	22.63±1	25.56±0.35	10.43±0.95	16.62±7.4
25	29.57±0.89	36.04±0.93	16.64±2.03	24.29±8.38
50	37.52±1.49	43.19±1.42	25.15±1.68	33.26±7.19
100	45.4±1.44	57±1.54	30.11±0.83	40.62±10.87
200	51.7±2.47	65.54±1.86	36.91±1.3	47.09±11.07
Total	30.3±14.89	37.41±18.07	18.31±12.2	25.55±16.48
IC_{50} µg/ml	171.31±13.93	70.33±5.21	>200	

Antioxidants play an important role in diminishing the harmful effects of ROS that correlated damage to cell membranes, enzymes, and DNA molecules. Oxidative stress is a crucial factor that contributes to the high mortality rates associated with dysregulation of the immune system, which in turn leads to a number of diseases. The immune system is particularly sensitive to the effects of oxidative stress (Valko *et al*., 2007). Antioxidants may also modulate cellular activity by inhibiting or changing the signal transduction of inflammatory cytokines and endotoxin at the onset of inflammatory reactions (Victor et al., 2004). However autoimmune diseases such as rheumatoid arthritis are characterized by an increase in the activity of leucocytes and fibroblasts that are responsible for the production of ROS. Van Vugt and coauthors (2008) described the Antioxidants that are used effectively as adjuvant therapy in the treatment of autoimmune diseases.

The current investigation of antioxidant activity assays showed high potency for Dothideoxanthone as an antioxidant compound, and its effects increase with increasing its concentration due to the rise in the number couple electrons that enlarged the reduction ability. (Karadeniz, 2005). The behavior of Dothideoxanthone as an antioxidant and scavenging activity accepted by previous studies that explained the antioxidant properties of xanthones compounds (Huang *et al*., 2012; Blanco-Ayala *et al*., 2013; and Gondokesumo et al., 2019).

Conclusion

The present finding revealed the ability of endophytic fungal isolates to produce xanthone secondary metabolites and its effects as an immunosuppressant and antioxidant, therefore, this investigation indicated the ability of further studies to that xanthone compound and evaluation of their activity in medical and pharmaceutical applications.

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References

Alfartosy, E. H., AlSaadoon, A. H., & Abdullah, A. S. (2021). Screening of Some Iraqi Plants for Fungal Species Producing Paclitaxel Anti-Cancer Drug. Annals of the Romanian Society for Cell Biology, 25(6), 5209-5223.

Almanaa, T. N., Rabie, G., El-Mekkawy, R. M., Yassin, M. A., Saleh, N., & EL-Gazzar, N. (2021). Antioxidant, antimicrobial and antiproliferative activities of fungal metabolite produced by Aspergillus flavus on in vitro study. Food Science and Technology, 42.

Bálint, M., Bahram, M., Eren, A. M., Faust, K., Fuhrman, J. A., Lindahl, B., ... & Tedersoo, L. (2016). Millions of reads, thousands of taxa: microbial community structure and associations analyzed via marker genes. FEMS microbiology reviews, 40(5), 686-700.

Blanco-Ayala, T., Lugo-Huitrón, R., Serrano-López, E. M., Reyes-Chilpa, R., Rangel-López, E., Pineda, B., ... & Torres-Ramos, M. (2013). Antioxidant properties of xanthones from Calophyllum brasiliense: Prevention of oxidative damage induced by FeSO 4. BMC Complementary and Alternative Medicine, 13, 1-15.

Boonyaketgoson, S., Trisuwan, K., Bussaban, B., Rukachaisirikul, V., & Phongpaichit, S. (2015). Isoflavanone and xanthone derivatives from Dothideomycetes fungus CMU-99. Tetrahedron Letters, 56(9), 1057-1059.

Conforti, F., Modesto, S., Menichini, F., Statti, G. A., Uzunov, D., Solimene, U., ... & Menichini, F. (2011). Correlation between environmental factors, chemical composition, and antioxidative properties of caper species growing wild in Calabria (South Italy). Chemistry & biodiversity, 8(3), 518-531.

Dangroo, N. A., Singh, J., Dar, A. A., Gupta, N., Chinthakindi, P. K., Kaul, A., ... & Sangwan, P. L. (2016). Synthesis of α-santonin derived acetyl santonous acid triazole derivatives and their bioevaluation for T and B-cell proliferation. European Journal of Medicinal Chemistry, 120, 160-169.

Degruyter, J., Woudenberg, J. H. C., Aveskamp, M. M., Verkley, G. J. M., Groenewald, J. Z., & Crous, P. W. (2013). Redisposition of Phoma-like anamorphs in Pleosporales. Studies in Mycology, 75(1), 1-36.

Fadiji, A. E., & Babalola, O. O. (2020). Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects. Frontiers in Bioengineering and Biotechnology, 8, 467.

Frisvad, J. C., Andersen, B., & Thrane, U. (2008). The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycological research, 112(2), 231-240

Fujimoto, H., Asai, T., Kim, Y. P., & Ishibashi, M. (2006). Nine constituents including six xanthone-related compounds isolated from two ascomycetes, Gelasinospora santi-florii and Emericella quadrilineata, found in a screening study focused on immunomodulatory activity. Chemical and pharmaceutical bulletin, 54(4), 550-553.

García-Carrasco, M., Jiménez-Hernández, M., Escárcega, R. O., Mendoza-Pinto, C., Galarza-Maldonado, C., Sandoval-Cruz, M., ... & Cervera, R. (2009). Use of rituximab in patients with systemic lupus erythematosus: an update. Autoimmunity reviews, 8(4), 343-348.

Gautam, V. S., Singh, A., Kumari, P., Nishad, J. H., Kumar, J., Yadav, M., ... & Kharwar, R. N. (2022). Phenolic and flavonoid contents and antioxidant activity of an endophytic fungus Nigrospora sphaerica (EHL2), inhabiting the medicinal plant Euphorbia hirta (dudhi) L. Archives of Microbiology, 204(2), 140.

Gondokesumo, M. E. (2019). Xanthones analysis and antioxidant activity analysis (Applying ESR) of six different maturity levels of mangosteen rind extract (Garcinia mangostana Linn.). Pharmacognosy Journal, 11(2), 369-373.

Gouda, S., Das, G., Sen, S. K., Shin, H. S., & Patra, J. K. (2016). Endophytes: a treasure house of bioactive compounds of medicinal importance. Frontiers in microbiology, 7, 1538.

Gunter, N. V., Teh, S. S., Lim, Y. M., & Mah, S. H. (2020). Natural xanthones and skin inflammatory diseases: multitargeting mechanisms of action and potential application. Frontiers in pharmacology, 11, 594202.

Huang, Z., Yang, J., Cai, X., She, Z., & Lin, Y. (2012). A new furanocoumarin from the mangrove endophytic fungus Penicillium sp. (ZH16). Natural Product Research, 26(14), 1291-1295.

Ibrahim, M., Oyebanji, E., Fowora, M., Aiyeolemi, A., Orabuchi, C., Akinnawo, B., & Adekunle, A. A. (2021). Extracts of endophytic fungi from leaves of selected Nigerian ethnomedicinal plants exhibited antioxidant activity. BMC Complementary Medicine and Therapies, 21(1), 1-13.

Kalala, W., Mwakigonja, A., Maregesi, S., Msengwa, Z., & Mahunnah, R. (2015). Brine shrimp lethality and acute oral toxicity of Commiphora swynertonii (Burrt) Exudate. Pyrex J Med Plant Res, 1(3), 010-8.

Karadeniz, F., Burdurlu, H. S., Koca, N., & Soyer, Y. (2005). Antioxidant activity of selected fruits and vegetables grown in Turkey. Turkish Journal of Agriculture and Forestry, 29(4), 297-303.

Khattab, A. R., & Farag, M. A. (2022). Marine and terrestrial endophytic fungi: A mine of bioactive xanthone compounds, recent progress, limitations, and novel applications. Critical Reviews in Biotechnology, 42(3), 403-430.

Li, J., Tu, Y., Tong, L., Zhang, W., Zheng, J., & Wei, Q. (2010). Immunosuppressive activity on the murine immune responses of glycyrol from Glycyrrhiza uralensis via inhibition of calcineurin activity. Pharmaceutical biology, 48(10), 1177-1184.

Liu, H., Chen, S., Liu, W., Liu, Y., Huang, X., & She, Z. (2016). Polyketides with immunosuppressive activities from mangrove endophytic fungus Penicillium sp. ZJ-SY2. Marine Drugs, 14(12), 217.

Lü, J. M., Lin, P. H., Yao, Q., & Chen, C. (2010). Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. Journal of cellular and molecular medicine, 14(4), 840-860.

Manganyi, M. C., & Ateba, C. N. (2020). Untapped potentials of endophytic fungi: A review of novel bioactive compounds with biological applications. Microorganisms, 8(12), 1934.

Mirhendi, S. H.; Adin, H.; Shidfar, M. R.; Kordbacheh, P.; Hashemi, S. J. and Moazeni, M. (2008). Identification of pathogenic Candida species: PCR-fragment size polymorphism (PCR-FSP) method. TUMJ. 66(9):639-645

Negi, J. S., Bisht, V. K., Singh, P., Rawat, M. S. M., & Joshi, G. P. (2013). Naturally occurring xanthones: chemistry and biology. Journal of Applied Chemistry, 2013, 1-9.

Nuraini, F. R., Setyaningsih, R., & Susilowati, A. (2019, July). Antioxidant activity of bioactive compound produced by endophytic fungi isolated from endemic plant of South Kalimantan Mangifera casturi Kosterm. In AIP Conference Proceedings (Vol. 2120, No. 1, p. 080013). AIP Publishing LLC.

Omomowo, I. O., Amao, J. A., Abubakar, A., Ogundola, A. F., Ezediuno, L. O., & Bamigboye, C. O. (2023). A review on the trends of endophytic fungi bioactivities. Scientific African, e01594.

Rasheed, A., & Azeez, R. F. A. (2019). A review on natural antioxidants. Traditional and Complementary Medicine, 1-24.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free radical biology and medicine, 26(9-10), 1231-1237.

Rimbawan, F., & Pinus Jumaryatno, A. F. (2018). Isolation Endophytic Fungi from Ficus carica L. as Antibiotic Producer against Staphylococcus aureus.

Rudrapal, M., Khairnar, S. J., Khan, J., Dukhyil, A. B., Ansari, M. A., Alomary, M. N., ... & Devi, R. (2022). Dietary polyphenols and their role in oxidative stress-induced human diseases: insights into protective effects, antioxidant potentials and mechanism (s) of action. Frontiers in pharmacology, 13, 283.

Salman, Z., Yu-Qing, J., Bin, L., Cai-Yun, P., Iqbal, C. M., Atta-ur, R., & Wei, W. (2019). Antioxidant nature adds further therapeutic value: an updated review on natural xanthones and their glycosides. Digital Chinese Medicine, 2(3), 166-192.

Smith, J. M., Nemeth, T. L., & McDonald, R. A. (2003). Current immunosuppressive agents: efficacy, side effects, and utilization. Pediatric Clinics, 50(6), 1283-1300.

Soares, J. X., Loureiro, D. R., Dias, A. L., Reis, S., Pinto, M. M., & Afonso, C. M. (2022). Bioactive marine xanthones: A review. Marine Drugs, 20(1), 58.

Song, X. Q., Zhang, X., Han, Q. J., Li, X. B., Li, G., Li, R. J., ... & Lou, H. X. (2013). Xanthone derivatives from Aspergillus sydowii, an endophytic fungus from the liverwort Scapania ciliata S. Lac and their immunosuppressive activities. Phytochemistry Letters, 6(3), 318-321.

Song, X. Q., Zhang, X., Han, Q. J., Li, X. B., Li, G., Li, R. J., ... & Lou, H. X. (2013). Xanthone derivatives from Aspergillus sydowii, an endophytic fungus from the liverwort Scapania ciliata S. Lac and their immunosuppressive activities. Phytochemistry Letters, 6(3), 318-321.

Spatafora, J.W.; Chang,Y.; Benny, G.L.; Lazarus, K.; Smith, M. E.; Berbee, M. L.; Bonito, G.; Corradi, N.; Grigoriev, I.; Gryganskyi, A. and James, T.Y. (2016). A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. Mycologia,108(5):1028- 1046

Srinivasan, R. M. J. N., Chandrasekar, M. J. N., Nanjan, M. J., & Suresh, B. (2007). Antioxidant activity of Caesalpinia digyna root. Journal of Ethnopharmacology, 113(2), 284-291.

Srinivasan, R. M. J. N., Chandrasekar, M. J. N., Nanjan, M. J., & Suresh, B. (2007). Antioxidant activity of Caesalpinia digyna root. Journal of Ethnopharmacology, 113(2), 284-291.

Taufiq, M. M. J., & Darah, I. (2018). Fungal endophytes isolated from the leaves of a medicinal plant, Ocimum sanctum Linn and evaluation of their antimicrobial activities. African Journal of Microbiology Research, 12(26), 616-622

Tedersoo, L., & Nilsson, R. H. (2016). Molecular identification of fungi. Molecular mycorrhizal symbiosis, 299-322.

Tekpinar, AD, & Kalmer, A. (2019). Utility of various molecular markers in fungal identification and phylogeny. Nova Hedwigia, 109 (1-2), 187-224.

Udenigwe, C. C., Lu, Y. L., Han, C. H., Hou, W. C., & Aluko, R. E. (2009). Flaxseed protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. Food Chemistry, 116(1), 277-284.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology, 39(1), 44-84.

Van Gelder, T., Van Schaik, R. H., & Hesselink, D. A. (2014). Pharmacogenetics and immunosuppressive drugs in solid organ transplantation. Nature Reviews Nephrology, 10(12), 725-731.

Van Vugt, R. M., Rijken, P. J., Rietveld, A. G., Van Vugt, A. C., & Dijkmans, B. A. (2008). Antioxidant intervention in rheumatoid arthritis: results of an open pilot study. Clinical rheumatology, 27, 771-775.

Wanasinghe, D. N., bell, K. D., Jeewon, R., Crous, P. W., Wijayawardene, N. N., Jones, E. B. G., ... & Karunarathna, S. C. (2017). Phylogenetic revision of Camarosporium (Pleosporineae, Dothideomycetes) and allied genera. Studies in Mycology, 87(1), 207-256.

Ye, K., Lv, X., Zhang, X., Wei, P. P., Li, Z. H., Ai, H. L., ... & Liu, J. K. (2022). Immunosuppressive isopimarane diterpenes from cultures of the endophytic fungus Ilyonectria robusta. Frontiers in Pharmacology, 12, 3847.

Yefrida, Suyani, H., Alif, A., Efdi, M., and Aziz, H. (2018). Modification of Phenanthroline Method to determine Antioxidant Content in Tropical Fruits Methanolic Extract. Research Journal of Chemistry and Environment, 22 (4), 28-35.

Zhang, X. Y., Liu, Z. L., Sun, B. D., Niu, S. B., Wang, M. H., Tan, X. M., ... & Ding, G. (2018). Bioactive resorcylic acid lactones with different ring systems from desert plant endophytic fungus Chaetosphaeronema hispidulur. Journal of agricultural and food chemistry, 66(34), 8976-8982.