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PRODUCTION OF TAXOL BY *CLADOSPORIUM* SPECIES ISOLATED FROM AIR, OKRA LEAVES AND FRUITS OF GRAPEVINE

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A great attention was paid towards exploiting microorganisms for cheap production of different bioactive compounds including paclitaxel (taxol) as a highly effective and broad spectrum natural anticancer agent. In this study five species of *Cladosporium* isolated from outdoor air, okra leaves and grapevine fruits in Egypt were tested for their abilities to produce paclitaxel. The fungal species were identified morphologically and by molecular techniques (sequencing ITS region of rDNA). Sequences were deposited in the GenBank as *Cladosporium halotolerans* AUMC 11387 (GenBank accession No. MN826823), *C. limoniformis* AUMC 11301(GB: MN826827), *C. sinusum* AUMC 11340 (GB: MN826919), *C. spharospermum* AUMC 10865 (GB: MN826828), *C. xanthochromaticum* AUMC 11366 (GB: MN826822). The fungal strains were grown on potato dextrose broth (PDB) and the paclitaxel was extracted using ethyl acetate. The fungal taxol was detected by thin layer chromatography (TLC) and evaluated quantitatively by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Results showed that all tested species were able to produce taxol but in variable levels. The best producers were *C. halotolerans* (5.943ug/L) and *C. sphaerospermum* (3.074ug/L). The remaining *Cladosporium* species yielded lower quantities of taxol ranging from 0.410 to 1.844ug/L. The study introduces *Cladosporium* species as important candidates for taxol production.

Key words: Fungi, *Cladosporium*, Taxol, Anti-cancer compounds

Introduction

Taxol is one of the most valuable natural products used clinically for treatment of several cancer types. It was first discovered and isolated from the bark of pacific yew tree *Taxus brevifolia* (**Stierle *et al*., 1993**). Taxol has been well established and approved by Food and Drug Administration (FDA) as a very important effective chemotherapeutic agent against breast, lung and ovarian cancer and other human tissue proliferating diseases (**Kohler and Goldspiel, 1994 and Raj *et al*., 2014**). However, the supply of taxol from the yew tree

has been very limited since the commercial production of 1 kg of taxol from this tree requires 10 tons of this very slow-growing tree or 300 trees (**Elavarasi *et al*., 2012**).

On the other side, with increasing its applications in chemotherapy, the availability and cost of the drug will remain an important issue (**Kwon *et al*., 1998**). So, seeking for new ways of obtaining taxol is the key to protect this limited resource and reduce the cost of drug therapy (**Frense 2007 and Wang *et al*., 2007**). Successive scientific efforts were focused on the production of this drug from renewable and low cost available sources with high amount. In the past few decades, scientists have succeeded in the isolation of endophytic taxol-producing fungi from various sources (**Stierle *et al*., 1993; Stroble *et al*., 1996; Wang *et al*., 2007 and El-Sayed *et al*., 2018**). Fermentation processes using taxol-producing microorganisms are considered as efficient and sustainable ways to produce taxol (**Gond *et al*. 2014 and Choi *et al*. 2016**).

Since the isolation of the first taxol- producing fungus *Taxomyces andreanae* (**Stierle *et al*., 1993**), several studies have been carried out and recorded possibility of taxol production by various fungal species such as *Pestalotiopsis malicola* (**Bi *et al*., 2011**), *Fusarium oxysporum* (**Elavrasi *et al*., 2012**), *Botryodiplodia theobromae* (**Raja *et al*., 2008**), *Phyllosticta citricarpa* (**Senthil *et al*., 2008**), *Aspergillus niger* (**Zhao *at el*., 2008 and Li. *et al*., 2017**), *Aspergillus terreus* (**El-Sayed*et al*., 2018**), *Aspergillus flavipes* (**El-Sayed *et al*., 2019**), *Aspergillus fumigatus* and *Alternaria tenuissima* (**Ismaiel *et al*., 2017**) and *Cladosporium cladosporioides* (**Zhang *et al*., 2009; Miao *et al*., 2018 and El–Sabbagh *et al*., 2019**). **Also, Kusari *et al*. (2014)** reported more than 40 fungal genera had the ability to produce taxol by fermentation processes.

In order to lower the costs of drug therapy, the search for new sources of taxol is always a demand. Taxol produced from fungi raise the hope for commercial production of this drug (**Staniek *et al*., 2009**). So, the current study was designed to isolate and identify *Cladosporium* species from air, okra leaves and fruits of grapevine and to determine the potentiality of different strains of *Cladosporium* to produce taxol as a precious compound.

Material and Methods

1-Isolation and phenotypic identification of *Cladosporium* species

a- Airborne fungi:

These fungi were isolated using the settle plate technique as recommended by **Li *et al.* (2010).** Five Petri plates of 9 cm diameter containing malt extract agar medium were exposed to the open air for 15 minutes in the localities indicated in table (1). Plates were sealed brought back to the laboratory then incubated at 25^{o}C for 7 – 15 days during which the developing fungi were identified and counted. The growing *Cladosporium* isolates were purified and preserved for further investigations.

b- Fungi inhabiting plant leaves and fruits:

The direct plating technique was used to isolate fungi contaminating the surface of okra leaves and grapevine fruits cultivated in Sohag Governorate. The collected Leaves were washed with running tap water and disinfected with 70% ethanol followed by sterile distilled water then they were cut into small pieces (1cm^2). Segments of leaves were cultured in plates containing malt extract agar (5 segments per plate) as recommended by **Pitt and Hocking (2009)**. Cultures were incubated at 28ºC for 7-14 days and the developing *Cladosporium* species were picked, purified and kept for further investigations. Identification to species level was done on the basis of their macro- and microscopic characteristics (**Bensch *et al*., 2012**).

2- Molecular (genotypic) identification of *Cladosporium* species

The tested *Cladosporium* species were individually grown on PDA medium and incubated at 25° C for 10 days **(Pitt and Hocking 2009).** Cultures were sent to the Molecular Biology Research Unit, Assiut University for DNA extraction using Patho-gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. The fungal DNA was then sent to SolGent Company, Daejeon South Korea for polymerase chain reaction (PCR) and gene sequencing. PCR was performed using ITS1 (forward) and ITS4 (reverse) primers which were incorporated in the reaction mixture. Primers have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G -

3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT-3'). PCR products (amplicons) were sequenced in the sense and antisense directions using ITS1 and ITS4 primers with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture (**White *et al*., 1990**). Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

3-Fermentation conditions and taxol extraction

For each of the five *Cladosporium* species, 2L Erlenmeyer conical flask containing 750 mL of potato dextrose broth (PDB) was prepared. Flasks were inoculated with15 ml of spore suspension containing 1 x 10^6 spore/ml of 7-day-old culture of the tested species (one flask/each species). The inoculated flasks were incubated for 21 days at 28°C in static conditions (**Elavarasi *et al*., 2012**)

At the end of the incubation period, the cultures were homogenized at 10000 rpm using a homogenizer (Mienta) for 3 min then filtered through four layers of cheesecloth to remove the mycelia. Filtrates were individually centrifuged at 5000 rpm for 10 min. Reduction of fatty acids that may contaminate taxol in the culture was carried out by addition of 0.025% sodium carbonate with continuous agitation for 10 min, then centrifugation at 5000 rpm for 10 min. Two equal volumes of ethyl acetate were added to the supernatant and they were agitated for 30 min at 200 rpm. Using a separation funnel, the organic phase was collected. The solvent was evaporated under reduced pressure at 35ºC using a rotary vacuum evaporator and the residue was air dried. The dried residue was then used as a crude taxol for analysis (**Elavarasi *et al*., 2012**).

4-Thin layer chromatographic (TLC) analysis

Ready-made TLC plates (20 x 20 cm) were activated in an oven at 80°C for 15 min. Each sample of the crude taxol was dissolved in 1ml methanol and spotted on the TLC plate (10µl/spot). Two TLC plates were prepared, one was transferred to the glass jar (20×22 ×10 cm) containing 100 ml of chloroform: methanol (7:1 v/v) and the

second was transferred to another jar containing chloroform: hexane (8:2 v/v). Solvents were left to run to the end point of each plate (**Wang *et al*., 2000 and Elavarasi *et al*., 2012**).

The chromatographic properties of the fungal crude extracts were detected with different reagents after gentle heating and compared with those of the authentic taxol (Paclitaxel, Actavis, Italy). The fungal taxol with the same chromatographic mobility and properties giving the same color reaction with visualization reagents was recorded. Two visualization reagents were used. The first was a mixture of sulfuric acid-methanol (1:1, v/v), 110°C (SAM) which gives dark brown colour with taxol at 0.67 RF and the second was vanillin-sulfuric acid (0.2g:20ml, w/v) (VSA) which showed blue colour at the same RF value.

5-Liquid chromatography-mass spectrometry (LC-MS/MS) analysis

The produced taxol was assayed using LC-MS/MS (Agilent Technologies 6420 Triple Quad LC/MS) in Analytical Chemistry unit, Department of Chemistry, Faculty of Science, Assiut University. Each sample was dissolved in 1 ml of 90 % methanol and then filtered through a polymeric filter with a pore size of 0.2 µm diameter. Five µl of each sample was injected into Zorbax Eclipse XDB - C18 Analytical column (4.6 x 150) mm. A variable wavelength recorder set at 250 nm was used to detect the compounds eluted from the column. The detected taxol was quantified by comparing the peak area of the samples with that of the standard taxol.

Results

***Cladosporium* species identified in the present study:**

Based on morphological and molecular characteristics, five species of *Cladosporium* were identified. These are *C. halotolerans* (from outdoor air at Sohag Governorate), *C. limoniforme* (from leaves of Okra plants), and *C. sphaerospermum* (from outdoor air). The remaining two species of *Cladosporium* were recorded for the first time in Egypt and were identified as *C. sinuosum* (from fruits of grapevine) and *C. xanthochromaticum* (from outdoor air). Pure cultures of all

isolated strains were preserved in the culture collection of Assiut University Mycological Center. Sequencing results of the ITS region of rRNA gene for five strains were also deposited in GenBank **(**Table 1) and the phylogenetic tree was constructed as shown in figure (1). Based on BLAST search sequences, *C. limoniform*e, *C. halotolerans, C. sphaerospermum* and *C. xanthochromaticum* showed more than 99.20% identity with similar strains accessed from the GenBank. *C. sinuosum* showed 97.8% similarity with the type strain (gb: NR_119659T) as well as four additional strains of the same species. This species is also characterized morphologically by its sinuate conidiophores.

Taxol levels detected by thin layer chromatography:

All the fungal species studied showed good growth on potato dextrose broth at 28°C and the culture filtrate of each was harvested and extracted with ethyl acetate. The crude extracts were subjected to chromatographic analysis and showed dark brown colour on TLC plates after spraying with sulfuric acid-methanol (1:1, v/v) and blue colour when another set of plates were treated with vanillin-sulfuric acid (0.2 g: 20 ml). Accordingly, taxol was detected in the extracts of all the five fungal species but with variable levels. Two *Cladosporium* species namely *C. halotolerans* AUMC 11387 and *C. sphaerospermum* AUMC 10865 produced taxol in relatively high amounts (deep brown or deep blue colours according to reagents) compared with the other 3 species; *C. xanthochromaticum* AUMC 11366, *C. limoniforme* AUMC 11301 and *C. sinuosum* AUMC 11340 which showed faint colors indicating lower levels of taxol (Table 2).

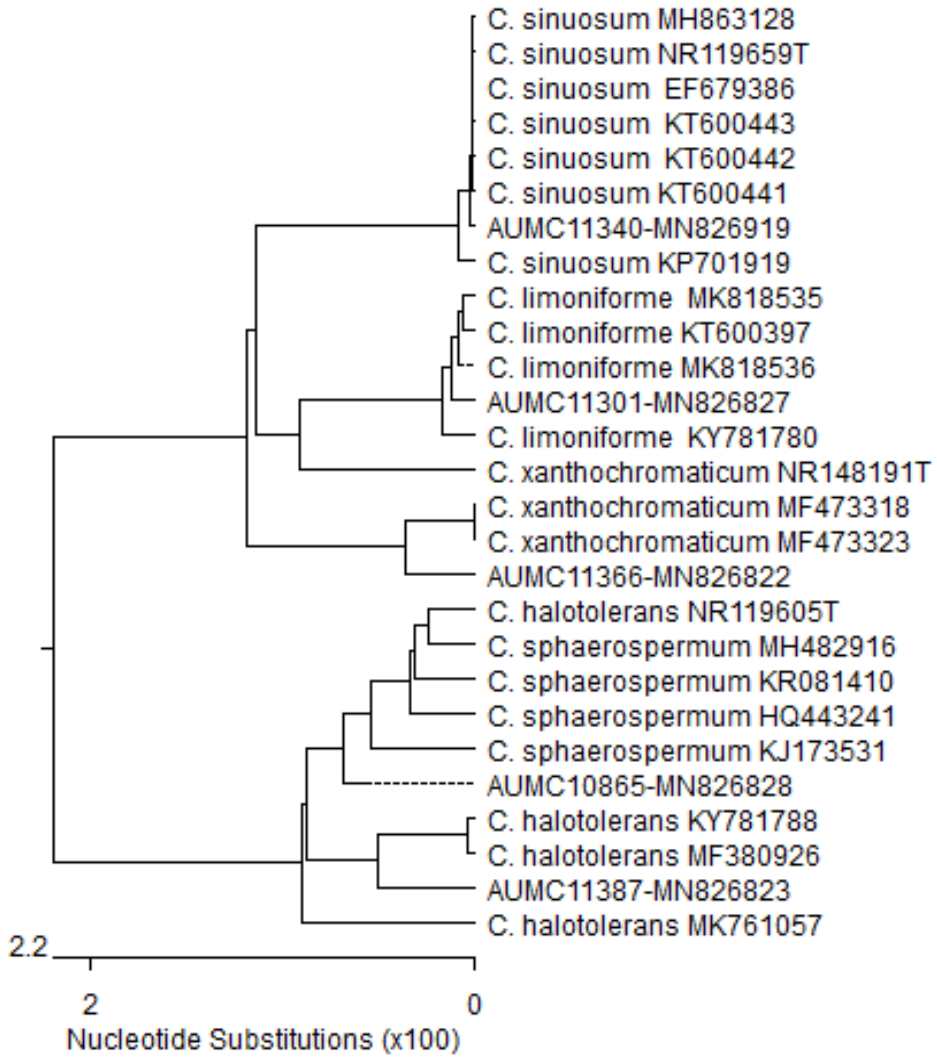

Figure 1: Phylogenetic tree based on ITS sequences of rDNA of *Cladosporium* strains isolated in the present study (given AUMC No.) aligned with closely related strains accessed from the GenBank

Table 1: Molecular identification of *Cladosporium* species aligned with closest strains accessed from the GenBank

Fungal species	AUMC No.	GenBank accession No.	Source and locality	Closest match from GenBank			
				Strain No.	Accession No.	Name	% Identity
C. limoniforme	11301	MN826827	Okra leaves, Sohag	C7	MK818535	*C. limoniforme*	99.46%
				CPC:12039	KT600397	*C. limoniforme*	99.61%
				C14	MK818536	*C. limoniforme*	99.46%
				QCC:M034/17	KY781780	*C. limoniforme*	99.66%
C. halotolerans	11387	MN826823	Air, Sohag	MEF 137	MF380926	*C. halotolerans*	99.46%
				QCC MO38/17	KY 781788	*C. halotolerans*	99.46%
C. sinuosum	11340	MN826919	Grapevine fruits, Sohag	CPC**:**11839	NR_119659T	*C. sinuosum*	97.90%
				CBS 121629	MH863128	*C. sinuosum*	97.90%
				CBS:393.68	KT600442	*C. sinuosum*	97.90%
				CPC:14000	KT600443	*C. sinuosum*	97.90%
				DTO 109-I2	KP701919	*C. sinuosum*	97.90%
C. sphaerospermum	10865	MN826828	Air, Beni Suef	KJ767065 = A1S1-1	KJ173531	*C. sphaerospermum*	98.90%
				RF94	KT151594	*C. sphaerospermum*	99.64%
C. xanthochromaticum	11366	MN826822	Air, Qena	DTO 323-E7	MF473323	*C. xanthochromaticum*	99.20%
				UTHSC DI-13-211	NR_148191T	*C. xanthochromaticum*	99.20%
				DTO:317-I2	MF473318	*C. xanthochromaticum*	99.20%

Table (2): Presumptive levels of taxol produced by *Cladosporium* species based on TLC analysis

Fungal species	Colour intensity		Level of taxol
	With SAM	With VAS	
C. limoniforme (AUMC 11301)	Faint Brown	Blue	L
C. halotolerans (AUMC 11387)	Dark brown	Dark blue	H
C. sinuosum (AUMC 11340)	Faint Brown	Blue	L
C. sphaerospermum (AUMC 10865)	Dark brown	Dark blue	H
C. xanthochromaticum (AUMC 11366)	Brown	Blue	M

H = high taxol production, M = moderate, L=low

Taxol levels detected by liquid chromatography- Mass spectrometry analysis

LC-MS/MS analysis was performed to confirm the presence of fungal taxol and to determine its concentration in the fungal extracts of the five fungal species. *Cladosporium halotolerans* AUMC 11387 and *C. sphaerospermum* AUMC 10865 yielded 5.943 and 3.074 µg/L taxol, respectively. *C. xanthochromaticum* AUMC 11366 produced 1.844 µg/L taxol. The remaining two strains of *Cladosporium* produced low quantities of taxol ranging from 0.410 µg/L (*C. sinuosum* AUMC 11340) to 0.820 µg/L *(C. limoniforme* AUMC 11301) as shown in table (3) and Figures (2-7).

Table (3): Concentration of taxol (ug/L) produced by *Cladosporium* species based on LC-MS/MS analysis

Fungal species	Retention time (min)	Concentration of taxol (µg/L)
Authentic taxol	2.136	25.00
C. halotolerans (AUMC 11387)	2.36	5.943
C. limoniforme (AUMC 11301)	2.160	0.820
C. sinuosum (AUMC 11340)	2.146	0.410
C. sphaerospermum (AUMC 10865)	2.136	3.074
C. xanthochromaticum (AUMC 11366)	2.13	1.844

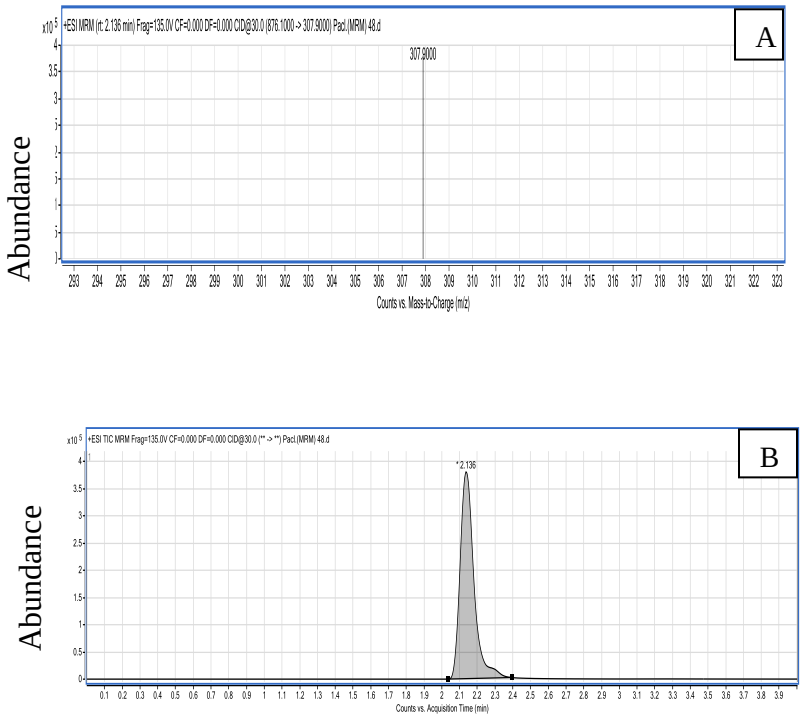

Figure 2: LC/MS/MS product ion spectrum of authentic taxol at 307.9 (m/z) (A) and retention time for authentic taxol (2.136 min) (B).

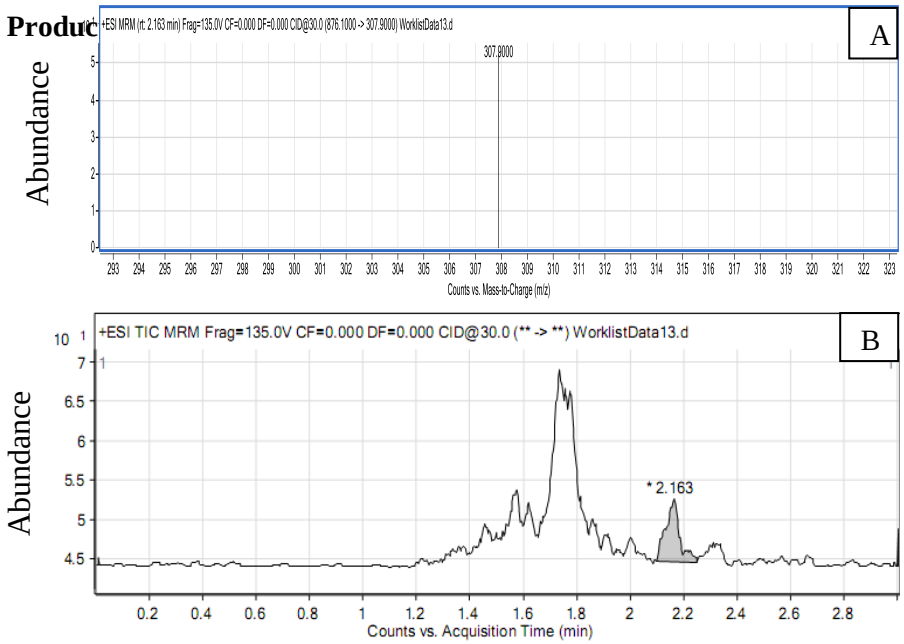

Figure 3: LC/MS/MS product ion spectrum at 307.9 (m/z) (A) and retention time (2.163 min) (B) of taxol produced by *C. halotolerans* AUMC 11387.

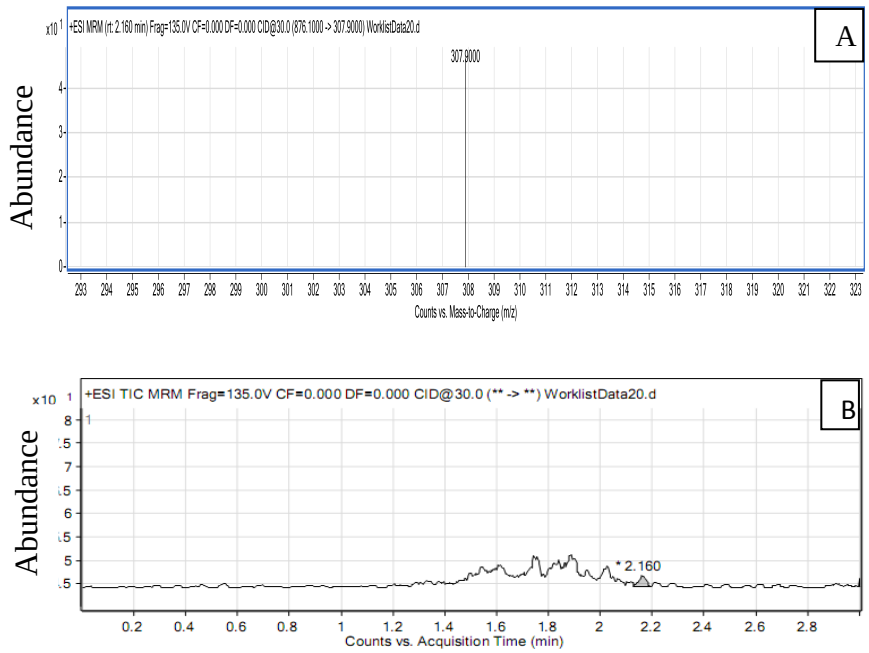

Figure 4: LC/MS/MS product ion spectrum at 307.9 (m/z) (A) and retention time (2.160 min) (B) for taxol produced by *C. limoniforme* AUMC 11301.

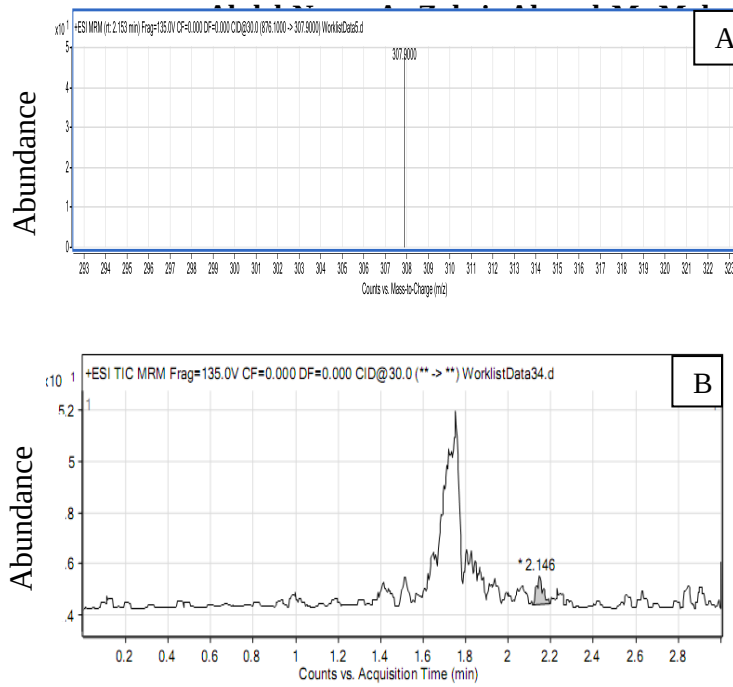

Figure 5: LC/MS/MS product ion spectrum at 307.9 (m/z) (A) and retention time (2.146 min) (B) for taxol produced by *C. sinuosum* AUMC 11340.

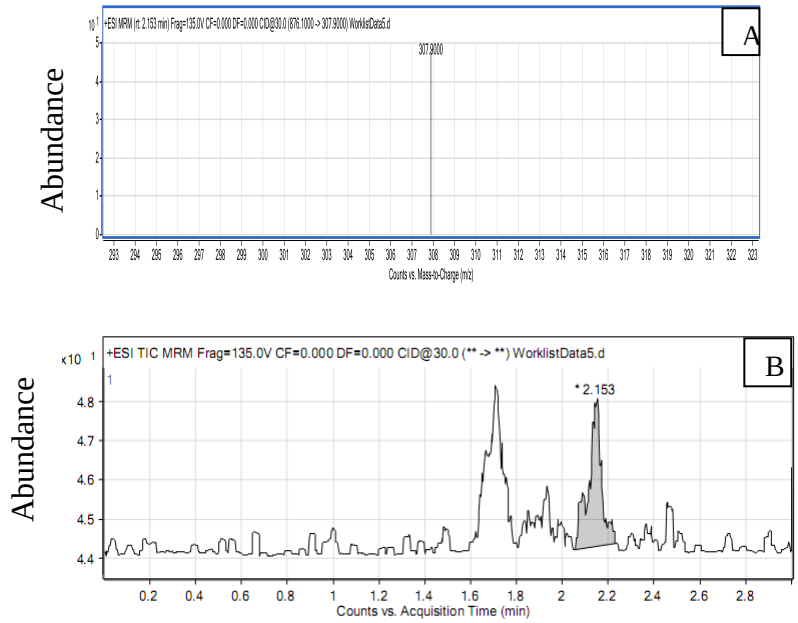

Figure 6: LC/MS/MS product ion spectrum at 307.9 (m/z) *(*A) and retention time (2.153 min) (B) for taxol produced by *C. sphaerospermum* AUMC 10865.

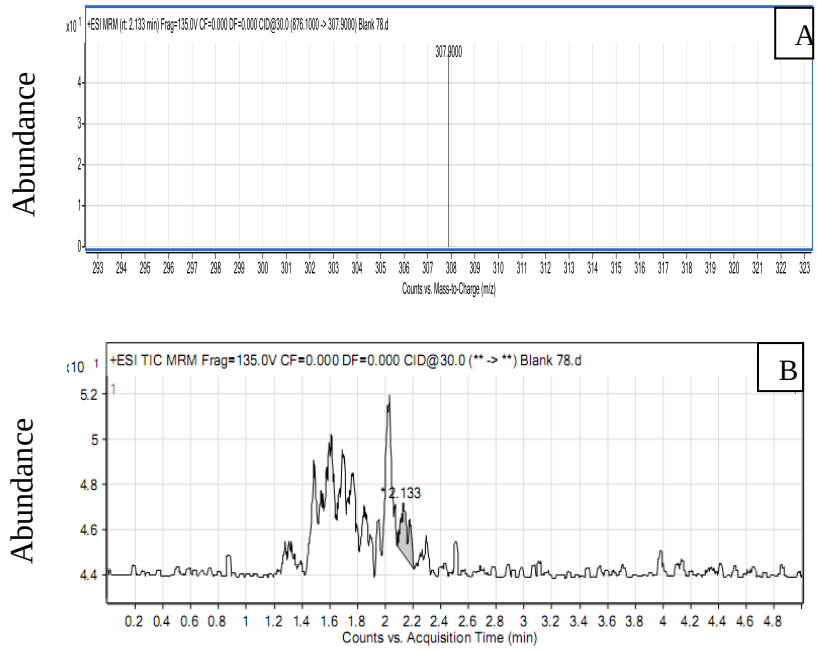

Figure 7: LC/MS/MS product ion spectrum at 307.9 (m/z) (A) and retention time (2.133 min) (B) for taxol produced by *C. xanthochromaticum* AUMC 11366.

Discussion

A total of five different species of *Cladosporium* were collected from of air as well as from fruits of grapevine and leaves of some plants and screened for taxol production. The results induced that all the tested strains have the ability to produce taxol but with variable levels. The fungal taxol was tentatively identified by comparison with the authentic taxol using TLC technique. It also gave same single peak when eluted from the C18 Analytical LC/MS/MS column, with about the same retention time as authentic taxol. *C. halotolerans* AUMC 11387 and *C. sphaerospermum* AUMC 10865 achieved high production of taxol at levels of 5.943 and 3.074 µg/L respectively. The remaining four species produced taxol in lower levels (0.410-1.844 µg/L). Several studies recorded the production of taxol by *C. cladosporioides* (**Zhang *et al.*, 2009; Gond *et al.*, 2014; Miao *et al.*, 2018** and **El-Sabbagh *et al.*, 2019**). In India**, Raj *et al.*, (2015)** reported that *C. oxysporum* had the ability to produce taxol. In Egypt, **Abo El-Maali *et al.* (2018)** recorded the production of 3.732 µg/L of taxol by *C. sphaerospermum* and the production was improved after incorporation of ammonium

acetate or salicylic acid (30.365 and 27.289 µg/L respectively). **Abd-Elsalam and Hashim (2013**) screened nineteen endophytic fungal genera for taxol production and found *Cladosporium* sp. had the ability to produce taxol in high levels.

Several other fungal species were recorded as taxol producers such as *Pestalotiopsis microspora* (**Stroble *et al.*, 1996**), *Tubercularia* sp. (**Wang *et al.*, 2000**), *Aspergillus niger* (**Zhao *et al.*, 2008**), *Botryodiplodia theobromae* (**Raja *et al.*, 2008**), *Phyllosticta citricarpa* (**Senthil *et al.*, 2008**), *Pestalotiopsis malicola* (**Bi *et al.*, 2011**), *Fusarium oxysporum* (**Elavarasi *et al.*, 2012 and Raj *et al.*, 2014**) and *Paraconiothyrium* sp. (**Soliman and Raizada 2018**). **Guo *et al.* (2006)** isolated an endophytic taxol producing fungus BT2 from *Taxus chinensis* var. *mairei* and found it produced taxol at 4-7 µg/L. **Sun *et al.* (2008)** recorded the ability of *Aspergillus fumigatus* for production of taxol at 5.553 µg/L. **El-Sayed *et al.* (2018)** were able to induced taxol production by *Aspergillus terreus* isolated from *Podocarpus gracilior.* They noticed that taxol yield was increased by 2.4 folds to 432 µg/L upon addition of surface sterilized leaves and cork tissues of *P. gracilior.* In India, **Sreekanth *et al.* (2011)** isolated taxol (5.38 µg/L) from *Gliocladium* sp. obtained from *Taxus baccata*.

No available literatures on production of taxol by *Cladosporium* species other than *C. cladosporioides*, *C. sphaerospermum* and C. *oxysporum*. Also, in this study the results recorded by TLC were confirmed using LC/MS/MS analysis. The taxol levels produced by *C. halotolerans* AUMC 11387, *C. sphaerospermum* AUMC 10865, *C. xanthochromaticum* AUMC 11366, *C. limoniforme* AUMC 11301 and *C. sinuosum* AUMC 11340 were determined as 5.943, 3.074, 1.844, 0.821 and 0.410 µg/L, respectively.

It is worthy to mention that two species of *Cladosporium* were recorded for the first time in Egypt from grapevine fruits and air. These are *C. sinuosum* AUMC 11340 and *C. xanthochromaticum* AUMC 11366. Moreover, these species in addition to *C. halotolerans* AUMC 11387 and *C. limoniforme* AUMC 11301 can be as considered new candidates able to produce taxol.

Taxol levels produced by *Cladosporium* species isolated in this study were comparatively lower than those recorded by some other

researches. **Zhang *et al*. (2009)** found that *C. cladosporioides* MD2 produced approximately 800 µg/L of taxol when it was cultured in potato dextrose broth at 25°C with 180 rpm for 10 days**. El-Sabbagh *et al.* (2019)** found that *C. cladosporioides* can produce 700 µg/L of taxol on potato dextrose broth medium at 25ºC, pH 5.5 after 10 days of incubation. **Gond *et al*. (2014)** at study the ability of fungi to produced taxol and found that *Cladosporium cladosporioides* MD2 have ability to produced taxol in high levels up to 800 µg/L. **Elavarasi *et al*. (2012)** recorded the production of 173.33 µg/L of taxol by *Fusarium oxysporium***.** While **El-Sayed *et al*. (2019)** recorded the production of 185 µg/L taxol by *Aspergillus flavipes.*

Conclusion

The current study introduced new *Cladosporium* species as candidates for taxol production. These species were identified on molecular basis as C. *sinuosum* and *C. xanthochromaticum.* Futher studies are needed to know the different factors required for maximization of taxol production by these fungal strains and to find suitable methods for purification of this important compound.

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إنتاج التاكسول بواسطة أنواع الكلادوسبوريوم المعزولة من الهواء وبعض النباتات المنزرعة

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هناك اهتمام كبير لاستغلال الكائنات الحية الدقيقة فى إنتاج مركبات نشطة بيولوجيًا وخاصة المركبات المضادة للاورام ومنها باكليتاكسيل (تاكسول) كعامل طبيعي مضاد للسرطان عالي الفعالية وواسع النطاق. تم في هذه الدراسة عزل خمسة أنواع من الكلادوسبوريوم من الهواء وأوراق نبات البامية وثمار العنب ودراسة مقدرتها على إنتاج مركب التاكسول (باكليتاكسيل) المضاد للسرطان. وقد اعتمد تشخيص الأنواع موضع الدراسة على أسس مورفولوجية وأيضا باستخدام التقنيات الجزيئية (تسلسل النيوكليوتيدات فى منطقة ITS من rDNA). وتم إيداع التسلسلات في بنك الجينات GenBankعلى النحو التالى: *Cladosporium halotolerans* AUMC11387 (accession No. MN826823), *C. limoniformis* AUMC11301(MN826827), *C. sinusum* AUMC11340 (MN826919), *C. spharospermum* AUMC10865 (MN826828), *C. xanthochromaticum* AUMC11366 (MN826822), وقد أمكن تنمية السلالات الفطرية على مرق دكستروز البطاطس (PDB) وتم استخلاص مركب الباكليتاكسيل باستخدام أسيتات الإيثيل. وتبع ذلك الكشف عن التاكسول بواسطة كروماتوغرافيا الطبقة الرقيقة (TLC) وأيضا باستخدام جهاز الفصل الكروماتوجرافى للسوائل المقترن بمطياف الكتلة (LC-MS/MS). أظهرت النتائج أن جميع الأنواع المختبرة لها القدرة على إنتاج التاكسول ولكن بمستويات مختلفة. وكانت أكثر الانوع إنتاجا للتاكسول كلادوسبوريوم هالوتوليرانس (5.943 ug/L) *C. halotolerans* و كلادوسبوريوم سفيروسبيرمم (3.074 ug/L) *C. sphaerospermum* ، أما الأنواع الأخرى من الكلادوسبوريوم فقد تراوح إنتاجها من التاكسول بين 0.410 و 1.844 ميكوجرام لكل لتر من الوسط الغذائى. تعتبر سلالات كلادوسبوريوم المعزوله والمختبرة فى هذا البحث إضافة جديدة للفطريات المنتجة للتاكسول المضاد للسرطان