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Production and Characterization of Taxol as Anticancer Agent from *Aspergillus terreus*

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Abstract

Taxol, a diterpenoid was initially isolated from the bark of *Taxus brevifolia,* approved by FDA in 1994 as a powerful drug for metastatic ovarian carcinoma, breast and lung cancer. However, due to limitations in the production of this drug based on this plant source, the productive potentiality of fungi of this compound opened a new avenue for its commercial production. In this study, among the twenty fungal isolates screened for Taxol production, *Aspergillus terreus* had the highest potentiality to produce Taxol (131.2 µg/ml). The productivity of Taxol by *A. terreus* has been maximized by nutritional optimization using inhibitors and growth regulators. The yield of Taxol by *A. terreus* was maximally obtained (0.663µg/ml) by growing the fungal isolate on potato dextrose broth medium, amended with addition of biotin at 150 µg/ml for 20 days. The chemical structure Taxol extracted of *A. terreus* has been verified by proton and carbon NMR, IR and UV analyses. The activity of *A. terreus* Taxol has been assessed towards multiple cell lines, displaying a strong anticancer activity. In conclusion, the productivity of Taxol by *A. terreus* has been greatly improved upon using biotin as chemical modulator, that open a new avenue for commercializing the Taxol yield by fungi.

Keywords: *Aspergillus terreus*; Taxol; Nutritional optimization, Inhibitors.

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INTRODUCTION

Taxol was firstly isolated and chemically identified by X-rays crystallography from the bark of Pacific Yew trees (*Taxus brevifolia*) (Wani *et al.*, 1971) and approved as a blockbuster anticancer drug for different cancerous types (Brown, 2003). The anticancer activity of Taxol arises from its specificity for binding with tubulin β-subunits, inducing tubulin polymerization, disrupting mitotic division of cancer cells (Straubinger *et al.*, 1993). Although Taxol is produced from the bark of *T. brevifolia*, its tiny yield and vulnerability to unpredicted fluctuation with the ecological conditions were the major challenges to this approach (Malik *et al.*, 2011; Wang *et al.*, 2011). However, semisynthetic process of 10-deacetylbaccatin III (DAB) intermediates isolated from the European Yew (*Taxus baccata*) is the current approach for production of Taxol, but the heterogeneity of DAB yield with ecological conditions of *T. baccata*, the need for wide cultivation area and breading time are the major limitation (Thomas and Polwart, 2003). Alternatively, endophytic fungi from *Taxus* spp with powerful potency for Taxol production, unlocked a new rout to scale-up the Taxol yield, for their fast growth, cost effectiveness, independency on climat changes, possibility for growing on bulk fermenters, and feasibility for genetic manipulation (Stierle *et al.*, 1993). With the higher applications of Taxol and its derivatives, the global demand of Taxol increases annually by approximately 10%, thus, Taxol sources are the current challenge to sustain its global affordability (El-Sayed *et al.*, 2017). Exploring of the fungal endophytes from Taxol producing and non-producing plants for ability to produce Taxol independent on their plant source and feasibility of metabolic manipulation raised the hope for industrial production of Taxol based on this technology. More than 250 endophytic fungal isolates were identified from *T. baccata* and about 20% of this population has the potentiality for Taxol production (Caruso *et al.*, 2000; Flores-Bustamante *et al.*, 2010). The biodiversity of fungal endophytes and their Taxol yield have been extensively reported (Strobel *et al.*, 1996; El-Sayed *et al.*, 2018a; Zein *et al.*, 2019; El-Sayed *et al.*, 2019; Artz *et al.*, 2011). Frequent reports claimed the medicinal plants of ethnopharmacological relevance could provide a fertile source for different therapeutics (El-Sayed *et al.*, 2019; El-Sayed *et al.*, 2018). So, exploring novel endophytic fungi from medicinal plants with promising, sustainable potency for Taxol production is the eventual objective. Among the plants of medicinal relevance, *Podocarpus* spp with ethnopharmacological significance were traditionally used for their activity against bacterial and fungal pathogens, and for cancer treatment (Abdillahi *et al.*, 2010; El-Sayed *et al.*, 2019) . Thus, the objective of this study was to screen the potentiality of endophytic fungi recovered from different medicinal plants, to produce Taxol. As well as to optimize the Taxol yield by the potent fungal isolate using different chemical inhibitors.

MATERIALS AND METHODS

Isolation and culturing of the endophytic fungi

Different plant types were collected from Sharkia province, Egypt, during June-August/ 2018, as a source of endophytic fungal isolates. These plants were Zea mays, *Solanum nigrum*, *Portulaco olerac*ea, *Convalvulus arrensis*, *Ocimum basilicum*, *Erchharnia crassipes*, *Rosa* sp, *Citrus xaurantium*, and *Ricinus communis*. The collected plant parts were cut into small segments (5mm) with sterile sharp blade then washed by sterile distilled water and then sterilized with 70% ethanol (v/v) for 2 min, then washed by 2.5% sodium hypochlorite (NaOCl) and dried on sterile filter paper (Xiong *et al.*, 2013). The plant parts were placed into the surface of potato dextrose agar (PDA) media with ampicillin (1µg/ml) as antibacterial agent. The plates were incubated at 28°C for 5-10 days. Sterile medium without plant segments was used as control to confirm the sterility of working area (El-Sayed *et al.*, 2018). After incubation, the developed hyphal tips of the fungi were picked and purified on fresh PDA plates, incubated for 5-10 days at 28°C, and the purified fungi were stored as slant culture at 4°C (Guo *et al.*, 2008).

Morphological and molecular identification of the recovered endophytic fungi

The purified fungal isolates were examined based on their microscopical properties according to (Raper and Fennell, 1965). The potent Taxol producing endophytic fungi were further confirmed based on their ITS rDNA sequence (El-Sayed *et al.*, 2019)(El-Sayed *et al.*, 2013)(El-Sayed *et al.*, 2015). The PCR reaction contained

10µl of 2' PCR master mixture (i-Taq™, Cat. # 25027, iNtRON Biotech), 1µl gDNA, forward and reverse primers (10 pmol/µl) and completed to 20µl. The PCR (Thermal Cycler 006, A&E Lab Co) was programmed to initial denaturation 94°C for 2 min, 35 cycles at denaturation 94°C for 20 s, annealing 55°C for 30 s, extension 72°C for 1 min, and final extension for 5 min at 72°C. The PCR products were checked by 2% agarose gel in 1' TBE buffer normalizing to the DNA ladder. The amplicons were purified and sequenced using the same primer sets. The sequences were BLAST searched non-redundantly on NCBI database, and the phylogenetic tree of target sequence was constructed with MEGA 6.0 software (Tamura *et al.*, 2011).

Chromatographic and spectroscopic analyses of extracted Taxol

The fungal cultures were grown on potato dextrose broth medium (PDB) (El-Sayed *et al.*, 2012), after incubation of the fungal cultures, Taxol was extracted (El-Sayed *et al.*, 2018 and 2019) and confirmed by TLC and HPLC. The extracted Taxol was analyzed by HPLC (Agilent Technology, G1315D) of C18 RP column (Eclipse Plus C18, 4.6*150mm), and methanol/ acetonitrile/water (25:35:40, v/v/v) as mobile phase, at flow rate 1 ml /min for 25 min and injection volume 20µl (Nims *et al.*, 2006). The fractions of Taxol were scanned from 200 to 500 nm by photoiodide array detector, the concentration and identity were determined from the retention time and absorption at 227 nm.

The Taxol chemical structure of Taxol was confined by ^{1}H NMR, ^{1}C NMR spectra comparing to authentic Taxol (JEOL, ECA-500, 500 MHz NMR), samples were dissolved in Deuterated chloroform (CDCL3), and the chemical shifts were given in ppm with coupling constant expressed in hertz (El-Sayed *et al.*, 2019).

The Infra-Red Spectra of extracted Taxol were analyzed using a JASCO, FTIR 6100 spectrophotometer. Samples of Taxol were ground in KBr pellets and pressed into discs by vacuum.

Nutritional optimization, and the effect of different inhibitors on Taxol yield

To investigate the optimal nutritional requirements of the potent fungi to maximize their Taxol yield, the influence of types of media in addition to the chemical inhibitors and activators were studied. The selected fungus was grown on Czapek's-Dox media (sucrose 30 g/L, sodium nitrate 2 g/L, K_2HPO_4 1 g/L, MgSO4 0.5 g/L, KCl 0.5g/L, FeSO4 0.01 g/L) (Onion's *et al.*, 1981), Potato Dextrose Broth (PDB) (potato extract 250 g, glucose 20 g, dissolved in one liter distilled water) (El-Sayed *et al.*, 2012), MID (El-Sayed *et al.*, 2019), malt extract (El-Sayed *et al.*, 2019) media.

The effect of different chemical compounds such as n-tosyl-l-phenylalanine, dinitro phenyl hydrazine, dimethylamino benzaldehyde, vitamin B_{12}, and biotin with different concentration (10 to 200µg/ml) on taxol production by the tested fungal isolate was evaluated. The cultures were filtered to remove any mycelia and Taxol was extracted as described above. The residues were re-dissolved in methanol and analyzed by chromatographic and spectroscopic methods, as mentioned above.

Antiproliferative activity of purified fungal Taxol

The activity of extracted Taxol was evaluated against breast carcinoma (MCF7). Cells at 10^3-10^5 per well were injected into the 96-well plate, incubated for 24 hr. MTT reagent (Trevigen, Gaithersburg, USA) was added to each well, the plate was re-incubated for 2 hrs. Detergent reagent (Trevigen, Cat# 4890-25-02) was added to wells, the plate was left in dark for 4 hrs and then read at 570 nm in the plate reader. The average values of triplicates were determined and the average value for the blank was subtracted. The growth inhibition percentage was calculated using the formula percentage of cell inhibition= 100-{(At-Ab)/ (AcAb)}x100. Which, "At" is the O.D of test compound, "Ab" is O.D of blank, and "Ac" is O.D of control.

Statistical Analysis

All experiments were conducted in biological triplicates and the results were expressed by mean ±STDEV. The data were analyzed by one-way ANOVA with Fisher's Least Significant Difference of post hoc test.

RESULTS AND DISCUSSION

Isolation and identification of the endophytic fungal isolates

Twenty fungal isolates were isolated and recovered as endophytes inhabiting the plants *Zea mays*, *Solanum nigrum*, *Portulaco oleracea*, *Convalvulus arrensis*, *Ocimum basilicum*, *Erchharnia crassipes*, *Rosa* sp, *Citrus xaurantium*,

Ricinus communis. Morphologically, the purified endophytic fungal isolates were identified according to universal keys. These fungal isolates were belonging to three genera; *Aspergillus, Fusarium, Penicillium*. *Aspergillus* represents seventeen isolates; *A. flavipes* (no.1), *A. niger* (no.5), *A. terreus* (no.3), *A. flavus* (no.7). *Fusarium* represented by two isolates (*F. solani* and *F. oxysporum*) and *penicillium* represents one isolate (*P. chrysogenum*) as shown in Table 1**.**

Spectroscopic and Thin layer chromatography analyses of extracted Taxol

The extracted Taxol from twenty fungal isolates was measured at wavelength 227 nm. Among these isolates, only eight fungal isolates displayed positive results, giving maximum absorption at ב227 nm, similar to authentic Taxol (Table 1). To confirm the spectroscopic assay, the samples were spotted on TLC chromatography comparing to authentic Taxol as described in Materials and Methods. The eight fungal isolates namely *A. flavipes, A.flavus*1, *A. flavus* 2, *A. flavus* 6, *A. terreus* 1, *A. terreus* 2, *A. terreus* 3, *P. chrysogenum* showed distinct spot under UV illumination at 254 nm with Rf value 0.7 which was identical to authentic taxol as showed in Fig. 1, with bluish spot when TLC sheets sprayed with vanillin/ sulphuric acid reagent. From these fungal isolates, *A. terreus* 1 displayed the highest yield of Taxol (131.7µg/ml) and the potent taxol spot.

HPLC, NMR and IR analyses of extracted Taxol

To investigate the presence of Taxol, fungal Taxol and authentic Taxol were subjected to HPLC analysis where authentic Taxol gave a peak with a retention time 1.8 min similar to fungal Taxol which gave a peak with retention time 1.89 min (Fig. 2). For further confirmation of the chemical structure of Taxol, the ^{1}H NMR analysis was performed where the signals of ^{1}H NMR for the fungal extract was found to be consistent to

Table 1. Spectroscopic and chromatographic analyses of the twenty fungal isolates

Isolate No.	Host Plant	Fungal isolate	Taxol Conc. (µg/ml)	TLC
1	*Zea mays*	*Aspergillus niger*1	4.92 ghi	++
2		*Aspergillus niger*2	52.12 d	-
3		*Aspergillus niger*3	12.38 def	-
4		*Aspergillus niger*4	24.18 fg	-
5	*Ricinus communis*	*Aspergillus niger*5	17.49 abc	+
6		*Aspergillus flavipes*	28.8 abc	-
7	*Solanum nigrum*	*Aspergillus flavus*	120.18 a	++
8		*Aspergillus flavus*2	119.0 ghi	+++
9		*Aspergillus flavus*3	28.8 ghi	-
10	*Portulaco oleracea*	*Aspergillus flavus*4	1.7 fgh	-
11		*Aspergillus flavus*5	4.7 ghi	-
12		*Aspergillus flavus*6	103.8 fgh	+
13	*Convalvulus arrensis*	*Aspergillus flavus*7	1.4 fghi	-
14		*Aspergillus flavus*	33.1 fgh	-
15	*Ocimum basilicum*	*Aspergillus terreus*1	131.9 a	+++
16		*Aspergillus terreus*2	106.2 fgh	+++
17	*Erchharnia crassipes*	*Aspergillus terreus*3	44.2 fghi	+++
18		*Fusarium oxysporum*	36.1 fghi	+
19	*Rosa* sp	*Fusarium solani*	14.6 fghi	+
20	*Citrus xaurantium*	*Penicillium chrysogenum*	93.8 fghi	++

- Negative Taxol producer, + Mild Taxol producer, ++ Higher Taxol producer, +++ Strong Taxol producer
values given are means
- The mean values followed by different letters a, b, c with in the same column are significantly different (ONE Way ANOVA, Tukey HSD test, $p \leq 0.05$).
*** means highly significant.
-LSD: the least significant difference.

the authentic taxol, the signals were distributed at 1.0 and 8.0 ppm. The proton signals appeared in the region between 1.0 and 2.5 ppm matching to methyl, acetate and acetylene groups (Kumaran *et al.*, 2008; El-Sayed *et al.*, 2019). Signals of most of protons in the taxane skeleton and their side chains protons were appeared in the region between 2.0 and 7.0 ppm while the signals for aromatic moieties corresponding to the benzoate (2-OCOph), phenyl (C3'-ph) and benzamide (3'NH-COph) groups were determined at 7.0 and 8.4 ppm (Chmurny *et al.*, 1992; Zhang *et al.*, 2009; Strobel *et al.*, 1996).

FT-IR analysis, Taxol of *A. terreus* displayed similar FT-IR peaks of standard Taxol as shown in Fig. 2. The peaks at 3406 and 3393 cm^{-1} were assigned for hydroxyl (OH) and amide (±C (O) NH±) groups stretch (Kumaran *et al.*, 2008). However, the peaks at 2923, 1729 and 1604 cm^{-1} were assigned to the aliphatic CH stretch, ester groups stretch and aromatic rings stretch (Das *et al.*, 2017). The COO stretching frequency was peaked at 1268 cm^{-1}, while the peak at 1029 cm^{-1} was allocated for the aromatic C, H bends.

Table 2. Effect of different chemical compounds on Taxol yield by *A. terreus*

Compound	Conc. (µg/ml)	Taxol conc. (µg/ml)	TLC	Increasing fold
Control	0	163.9±9.6 ^{ghi}		-
N-tosyl-L-	10	286.9±34 ^{efgh}	+	1.7
phenyl	50	215.7±5.6 ^{fghi}	+	1.3
alanine	100	331.8±1.7 ^{def}	+	2
	150	162.3±3.7 ^{hi}	-	-
	200	318.5±2.4 ^{ef}	+	1.94
Dinitrophenyl	10	375.8±11.6 ^{cde}	+	2.29
hydrazine	50	307.6±75.2 ^{efg}	+	1.87
	100	605.6±2.8 ^{ab}	+++	3.6
	150	471.5±2.5 ^{bcd}	++	2.8
	200	128.1±8.4 ^{i}	-	-
Dimethyl amino	10	376.6±8.9 ^{cde}	+	2.29
benzaldehyde	50	570.8±42.6 ^{ab}	++	3.48
	100	514.2±7.5 ^{bc}	++	3.13
	150	341.4±7.4 ^{def}	+	2.08
	200	320.9±6.4 ^{ef}	+	1.95
Vitamin B12	10	379.7±71.2 ^{cde}	-	2.3
	50	571.8±90.5 ^{ab}	++	4.17
	100	510.5±95.5 ^{bc}	++	3.11
	150	343.4±11.61 ^{def}	+	2.8
	200	325.8±68.9 ^{ef}	+	1.98
Biotin	10	361.6±63.5 ^{de}	+	2.2
	50	347.6±85.9 ^{def}	+	2.2
	100	410.8±22.9 ^{cde}	++	2.5
	150	663.8±0.57 ^{a}	+++	4.04
	200	476.7±76.8 ^{bcd}	+++	2.9
P	-	***(0.0000)		
$LSD_{0.05}$	-	74.88		

-values given are means

- The mean values followed by different letters a, b, c with in the same column are significantly different (ONE Way ANOVA, Tukey HSD test, $p \leq 0.05$).

*** means highly significant .

-LSD: the least significant difference.

Nutritional optimization of *Aspergillus terreus* to maximize their Taxol yield

Different types of media

Aspergillus terreus was cultivated on different media (PDB, MID, DOX, Malt extract). From the obtained results (Fig. 3), *A. terreus* gave the highest Taxol yield by growing on PDB media (132µg/ml) followed by malt extract media (128.47µg/ml), with distinct corresponding Taxol spots on the TLC plates. However, the yield of Taxol by *A. terreus* was significantly low, with undetectable spots on TLC plates, under the standard conditions by growing on Czapek's-Dox and MID media. Consistently, PDB supports the maximum Taxol yield by *A. terreus* (El-Sayed *et al.*, 2018b) and *A. flavipes* (El-Sayed *et al.*, 2019a).

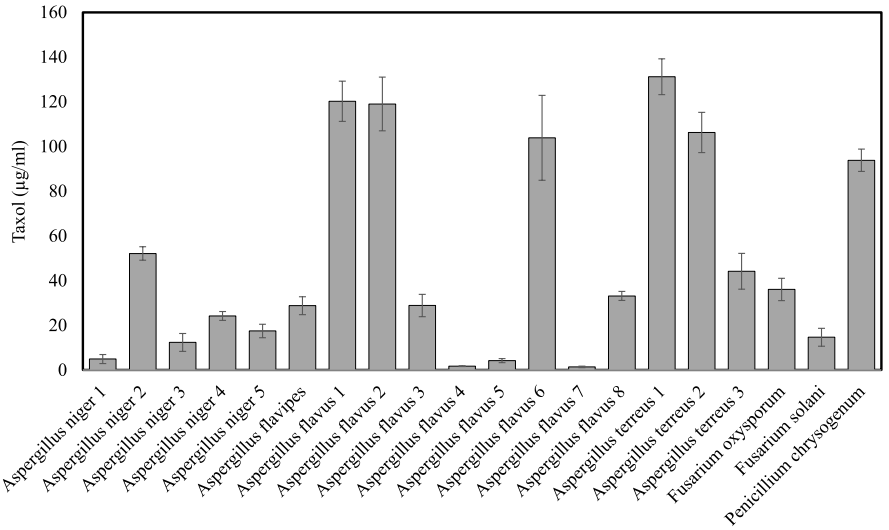

Fig. 1. Screening for Taxol producing potency among the recovered endophytic fungi

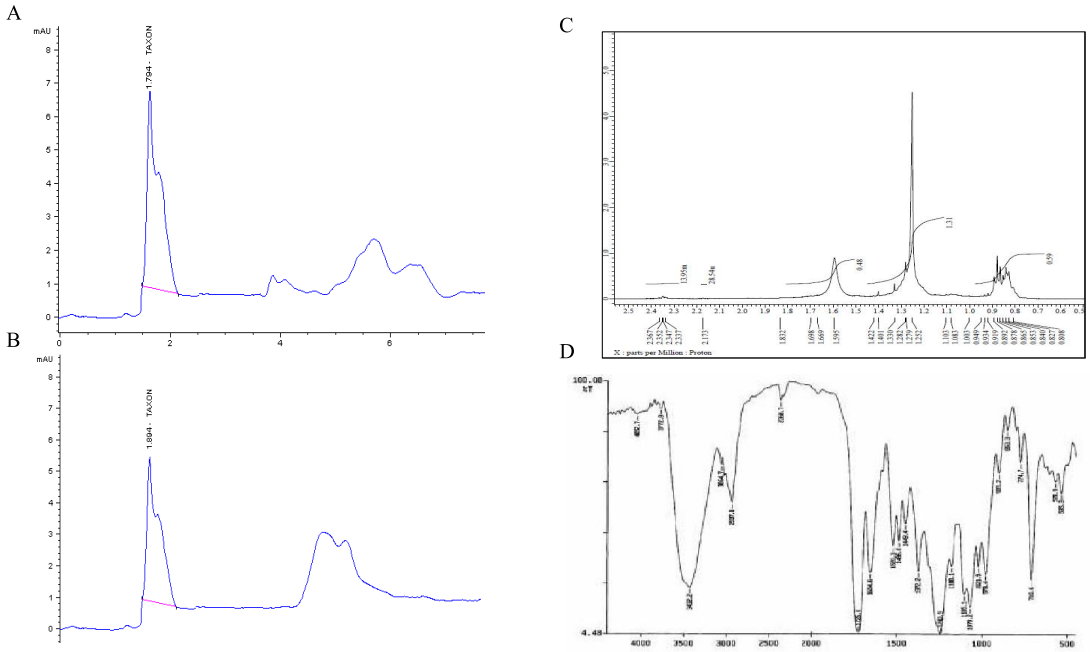

Fig. 2. Chromatographic and Spectral analyses of the extracted Taxol from A. terreus. HPLC chromatogram of authentic Taxol. (A), *A. terreus* extracted Taxol (B). NMR spectrum (C) and FT-IR spectrum (D) of *A. terreus* Taxol

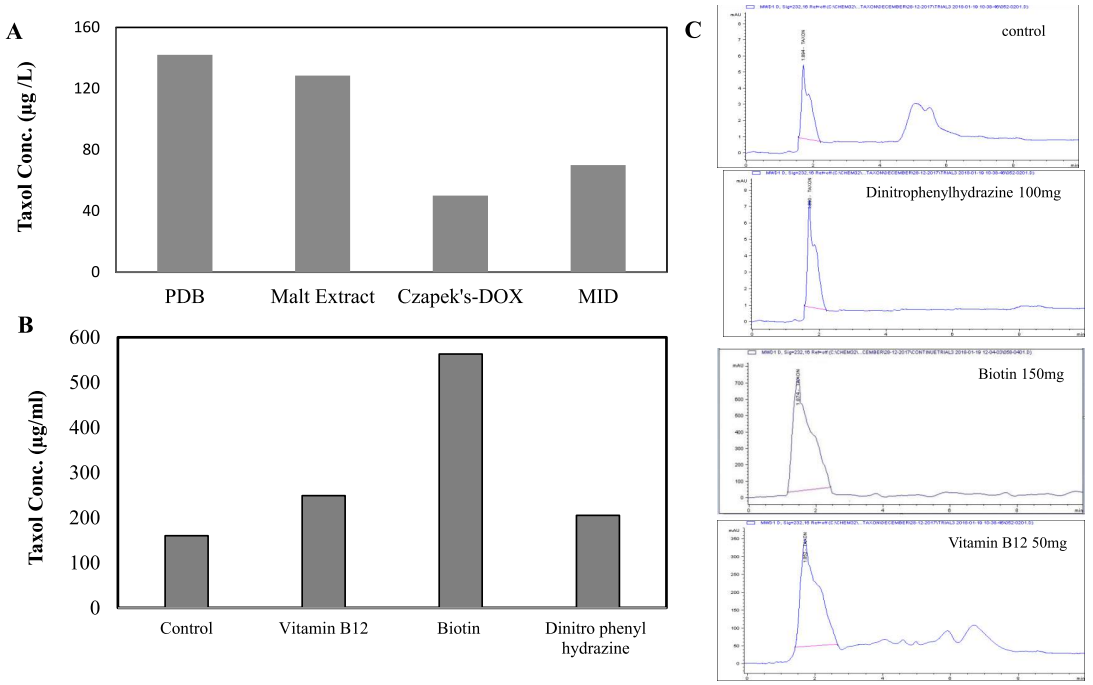

Fig. 3. Taxol yield of *A. terreus* by growing on different types of media and in response to different growth inhibitors. A, The fungal isolate was grown on different potato dextrose broth, malt extract, Czapek's-Dox and MID media (El-Sayed et al, 2018), and the Taxol was extracted and determined by HPLC. The cultures of *A. terreus* were amended with different inhibitors, incubated at standard conditions, then Taxol was extracted and quantified (B) from HPLC chromatogram (C).

Different chemical compounds

The effect of various inhibitors such as *N*-p-tosyl-l-phenylalanine, dinitrophenyl-hydrazine, dimethyl amino benzaldehyde, vitamin B12, Biotin at different concentration (10, 50, 100, 150, 200µg/ml) on Taxol production by *A. terreus* was evaluated. The fungal cultures were cultivated with each compound on PDB medium for 15 days at 28°C, then the Taxol yield was determined. From the results of UV spectroscopy and TLC chromatography, the Taxol yield by *A. terreus* in presence of biotin (150µg/ml), vitamin B12 (150µg/ml) and dinitrophenyl-hydrazine (100µg/ml) was significantly increased by about 3.5, 3.5 and 4.1 folds, respectively, comparing to control (Table 2), with *p* value was < 0.0001. Thus, among the tested compounds, biotin displayed a significant inducing activity on Taxol yield by *A. terreus* by about 4.1 folds. The Taxol yield by *A. terreus* was slightly increased in presence of vitamin B_{12}, dimethylamino benzaldehyde and dinitrophenyl hydrazine at 10µg/ml comparing to control. The taxol yield by *A. terreus* in response to these inhibitors was authenticated from the HPLC analysis (Fig. 4). From the obtained results, the Taxol yield by *A. terreus* was maximally increased

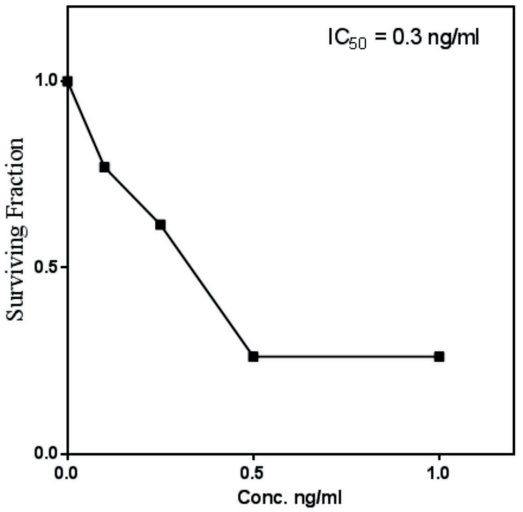

Fig. 4. Antiproliferative activity of extracted Taxol from *A. terreus* towards MCF-7 breast cell lines *in vitro*.

by incorporation of biotin, vitamin B12, and dinitrophenylhydrazine by 5, 2.2 and 1.6 folds, respectively, comparing to control.

Antiproliferative activity using MTT test

The antiproliferative activity of *A. terreus* Taxol was evaluated against MCF7-E (breast cancer cell). The IC_{50} value of *A. terreus* Taxol was 0.3 ng/ml towards MCF7-E (Fig. 4). The activity of extracted Taxol was noticed to be a dose-dependent manner towards the tested cell line. Coincident results authenticating the antiproliferative activity of *A. terreus* Taxol was documented in our previous studies (El-Sayed *et al.*, 2019c; El-Sayed *et al.*, 2018a and Zein *et al.*, 2019).

CONCLUSION

Aspergillus terreus an endophyte of *Ocimum basilicum* displayed the highest potency for Taxol production, among the recovered endophytic fungal isolates from different medicinal plants. The yield of Taxol from *A. terreus* has been increased by about 4 folds upon addition of biotin at 150µg/ml to the culture media after 5 days of cultural pre-incubation. The chemical identity of the extracted Taxol was validated by HPLC and spectroscopic analysis, as well as their anticancer activity was emphasized towards the MCF-7, regarding to authentic Taxol.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

ASAE and AEH designed and wrote the manuscript; MTE and HSN revise and edit the manuscript, EKY perform the experiments.

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ETHICS STATEMENT

The study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

DATA AVAILABILITY

The raw data required to reproduce these findings are available to download from https://www.researchgate.net/profile/Ashraf_El_Sayed.

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