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Paclitaxel production by endophytic fungus, *Neopestalotiopsis clavispor*a KY624416 and subsequent extraction of chitosan from fungal biomass wastes

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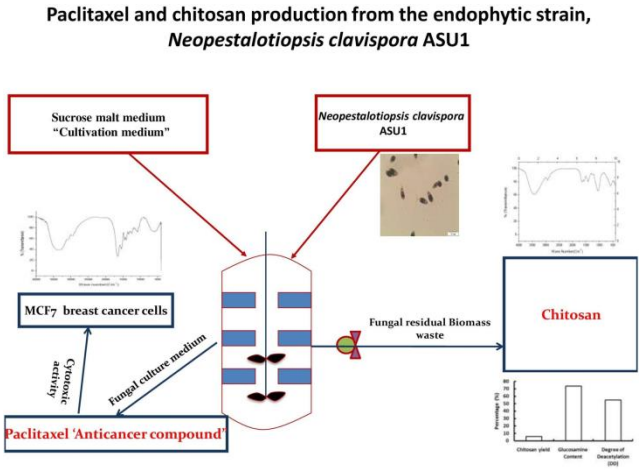
Graphical abstract



Abstract

Nowadays, breast cancer is considered to be one of the most prevalent disease worldwide, this initiated interest and growing demand for taxol production in large quantities. The limited availability of traditional taxol production from the Pacific yew trees has encouraged research into the development of taxol production from alternative sources. Thus, the current study aimed to investigate the chemopreventive effect of paclitaxel derived from endophytic fungus *Neopestalotiopsis clavispora* ASU1. The endophytic fungus *Neopestalotiopsis clavispora* ASU1(KY624416) showed potent productivity of paclitaxel recording 100.6 µg/l which was confirmed by HPLC, LC/Ms-Ms and FTIR analyses. *In vitro*, the extracted fungal taxol exerted a significant cytotoxic effect (P< 0.05) at 300 nM, revealed that the increase in paclitaxel concentration induced increasing in cell death. Furthermore, the present study provided a promising approach for coupling paclitaxel production technology by endophytic fungus *N. clavispora* with chitosan production from residual fungal biomass resulting from taxol extraction and therefore improve the feasibility and commercialization of taxol production. The chitosan yield represented 5.94% of residual fungal dry biomass. Also, fungal chitosan was characterized for the degree of deacetylation (DD) (54.60%), FTIR spectroscopy, reducing power activity (0.263±0.051 mg/ml) may be attributed to hydroxyl groups (OH), and amine groups. These results confirmed that fungi are promising alternative sources for chitosan with superior physiochemical characteristics for food and medical prospective applications.

Keywords: Anticancer, Chitosan, MCF7 cell line, *Neopestalotiopsis clavispora*, Paclitaxel.

1. Introduction

Paclitaxel is a natural diterpenic alkaloid compound and one of the most successful anticancer drugs (Brown, 2003; Suffness, 1995; Vasundhara *et al.*, 2016). It is used for the treatment of some cancer diseases, such as ovarian, breast, leukemias, head and neck carcinoma, lymphomas and lung cancers as well as Kaposi's sarcoma (Brown, 2003; Chakravarthi *et al.*, 2008; Croom Jr, 1995; Kharwar *et al.*, 2011; Vennila *et al.*, 2010). The mode of action of paclitaxel is to promote the assembly of microtubule-polymers and reduce the critical concentration of purified tubulin subunits necessary for polymerization into microtubules, leading to the failure of mitosis. Furthermore, it may alter other cellular functions, such as intracellular signaling, organelle transport and locomotion (Alexandre *et al.*, 2007; Weaver, 2014). Paclitaxel was isolated from the bark of *Taxus brevifolia,* the Pacific yew tree (Heinig *et al.*, 2013; Perdue, 1969; Wani *et al.*, 1971). But, unfortunately, very low quantities of produced taxol and the destruction of trees are considered the major obstacles associated with paclitaxel production from the Pacific yew tree (Gallego *et al.*, 2019). So, the produced amount of paclitaxel cannot meet the increasing demand for anticancer taxol compound on the market (Chakravarthi *et al.*, 2008), leads to increase the efforts to develop alternative sources of taxol production, such as chemical synthesis, but this is very expensive and gives low yields (Frense, 2007; Guenard

et al., 1993). This has encouraged researchers to explore other taxol candidates and develop technologies that generate a high yield of taxol from sustainable resources. Microorganisms are considered to be one of the most environmentally acceptable, relatively simple and cost-competitive paclitaxel feedstocks as they are characterized by a fast growth with high cell densities, easy scale-up, shear stress resistance, simple genetic manipulation, and dependable and unrestrained paclitaxel production. The last two aspects make microorganisms important requisites for industrial production and increase paclitaxel fermentation technology. Interestingly, over 30 taxol-producing fungi have been reported from previously published papers, the majority are the endophytes of *Taxus* spp. (Yuan *et al.*, 2006). Thus, endophytic fungal fermentation processes may be an alternative way to produce taxol (Wang *et al.*, 2000). With respect to *Pestalotiopsis*, several taxol-producing isolates were recovered from bald cypress in South Carolina (Strobel *et al.*, 1997). Also, it was produced from *Pestalotiopsis microspora*, *P. pausiceta*, *P. terminaliae* and *Chaetomella raphigera* isolated from *Taxus wallichiana*, *Taxodium distichum*, *Cardiospermum helicacabum*, *Terminalia arjuna* and *Aspergillus fumigatus* (Gangadevi and Muthumary, 2008; Gangadevi and Muthumary, 2009; Kumar *et al.*, 2019; Strobel *et al.*, 1996a; Strobel *et al.*, 1996b; Vennila *et al.*, 2010). Furthermore, there are several anticancer compounds isolated from endophytic fungi following paclitaxel, but no of them has had such effect on cancer chemotherapy as paclitaxel (Kharwar *et al.*, 2011).

Chitosan is a natural amino polysaccharide of D-glucosamine (GlcN) and N-acetyl-D-glucosamine (GlcNAc) (No *et al.*, 1989) and is a deacetylated distinctive form of chitin. Chitosan is characterized by polycationic, non-toxic, biodegradable and antimicrobial properties with numerous economic applications, especially in food, pharmaceutics and cosmetics (Morin-Crini *et al.*, 2019). Chitosan was produced commercially from the exoskeleton of crustaceans through insensitive chemical treatments. However, this is the main drawback in the chitosan extraction process and has limitations in industrial applications due to limited crustacean shells supply, expensive production process and inconsistent physicochemical characteristics (Wu *et al.*, 2005). These drawbacks nominate fungi to be a potentially promising source for chitosan production, as production and purification of chitosan from the fungal walls grown under controlled conditions offer a great advantage of being environmentally friendly and provides potential aspects for a reliable product (Dhillon *et al.*, 2013b; Abdel-Gawad *et al.*, 2017). As mentioned above, fungi are characterized by a fast growth with high cell biomasses, obtained by simple fermentation and cost-competitive processes, regardless of geographical location or season (Teng *et al.*, 2001).

The current study aimed to 1) isolate the endophytic fungus *Neopestalotiopsis clavispora* and investigate its capability for paclitaxel production from fungal culture. 2) maximize the economic importance of paclitaxel through the concomitant production of fungal chitosan from the fungal biomass residue. 3) outlined paclitaxel anticancer activity and cytotoxicity against cancer cell lines.

2. Materials and methods

2.1. Endophytic fungus isolation and identification

Avocado fruits (*Persea americana* L.) (10 samples) were collected from a market in Makkah city, Saudi Arabia in sterile polyethylene bags and transferred to the laboratory. One centimeter square from avocado fruits was washed by distilled water and surface sterilized using ethanol (70%) for 1 min and sodium hypochlorite (5%) for 3 min. the pieces were rinsed subsequently and transferred to agar plates containing potato dextrose agar medium (potato, 200 g/l; dextrose, 20 g/l; agar, 20 g/l, distilled water, 1000 ml) supplemented with chloramphenicol (250 mg/l) (Smith and Dawson, 1944). The plates were incubated at 28ºC for 7 days. The growing fungal isolate was subcultured in pure culture and kept on a PDA slant at 4°C for further studies. Microscopic slides were prepared and examined under a light microscope (Olympus CX 41 microscope) equipped with a digital camera (SC30 Olympus digital camera, Japan) for morphological identification.

The molecular identification of isolated fungus was performed in SolGent Company, Daejeon, South Korea by amplification of nuclear ribosomal RNA using primers ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). The sequence of 18S rRNA of a fungal strain containing 501 bases was first analyzed using the advanced BLAST search program on the NCBI website: http://www.ncbi.nlm.nih.gov/BLAST/T in order to assess the degree of DNA similarity. The nucleotide sequence of fungal strain ASU1 was deposited in the Genbank nucleotide sequence database under accession number KY624416. Phylogenetic tree derived from 18S rRNA gene sequence was generated using phylogeny.fr software (http://www.phylogeny.fr/) in comparison to 18SrRNA gene sequences from eight different standard fungal strains obtained from Genbank: *Phoma* sp. QC-2014b (KP330455), *Phoma herbarum* (EU754186), *Fusarium oxysporum* f. sp. *cepae* (GU166124), *Penicillium janthinellum* (GU981625), *Pestalotiopsis paeoniicola* (EU400221), *Neopestalotiopsis clavispora* (KR052094), *Pestalotiopsis* sp. LK2 (EU047942), *Neopestalotiopsis foedans* (KU593530) (Abdel-Sater *et al.*, 2016).

2.2. Paclitaxel production by endophytic fungus

2.2.1. Fungal inoculums preparation

Neopestalotiopsis clavispora ASU1, isolated from Avocado fruits and maintained in PDA (potato dextrose agar) at 4 °C, was used in this study. Spore suspension was used for inoculation of the flask cultures. Potato dextrose agar plates were inoculated with fungal spores and incubated for 7 days at 28 ± 1 °C. After growth, fungal hyphae were collected and blended with sterilized distilled water. One ml of the homogenous solution of fungal propagules was used for inoculation.

2.2.2. Culture medium and cultivation method for paclitaxel production

The flask culture sucrose malt medium contained (g/l): Sucrose, 50; malt extract, 17; peptone, 3; and chloramphenicol, 0.25. After sterilization, the medium was inoculated with 1 ml of spore suspension. 250 ml Erlenmeyer flasks containing 100 ml of sterilized medium were incubated on a rotary shaker (Environ 3597-1, Labline Instruments, USA) at 120 rpm at 28˚C for 10 days in the dark.

2.2.3. Extraction of paclitaxel from fungal culture

Neopestalotiopsis clavispora ASU1 was grown in submerged culture as described above, after 10 days the culture (medium-plus mycelium) was extracted twice with an equal volume of 95% methanol (Strobel *et al.*, 1996b). The filtrate with organic solvent was shacked over the night and dried in an evaporator under a vacuum at 65°C. The residue was dissolved in 1 ml methanol and analyzed by HPLC. Furthermore, the fungal biomass residue was collected and dried at 50°C up to constant weight and used for chitosan production.

2.3. Characterization of fungal paclitaxel

2.3.1. High-performance liquid chromatography (HPLC)

The extracted fungal paclitaxel was analyzed using Agilent HPLC (Agilent Technologies Series 1200, G1315DDAD, at the Analytical Chemistry Unit, Chemistry Department, Faculty of Science, Assiut University). With column Zorbax Extend C18, analytical (4.6 ×150 mm-5Micron) and mobile phase isocratic elution: A: methanol 70 %, B: milli-Q 30 %. The flow rate was 1.5 ml/min at 30 °C. The injection volume was 100 µL and the detector was DAD at 232 nm (Markeb *et al.*, 2016).

2.3.2. Liquid chromatography–MS-MS (LC/MS-MS)

After HPLC analysis of the extracted fungal paclitaxel, chromatographic analysis by LC/MS-MS was also performed using Agilent LC/Ms (Agilent Technologies 6420 Triple Quad LC/Ms, at the Analytical Chemistry Unit, Chemistry Department, Faculty of Science, Assiut University) for confirmation of paclitaxel concentration. 5 µl of fungal paclitaxel was injected into an Agilent LC/MS Triple Quad LC/Ms equipped with Zorbax Eclipse XDB-C18, analytical column (4.6 ×150 mm-5 Micron) and mobile phase B: Milli-Q elution 10 %, C: Methanol 70 %. The flow rate was 0.8 ml/min at 40 °C. Ms QQQ mass spectrometer system was operated as follows: The ion source is electrospray (ESI) positive ion mode; the gas temperature was 350 °C and the gas flow rate was set at 11 l/min; nebulizer was 45 psi and voltage setting on the capillary with the ion spray voltage was controlled at 4kV. Paclitaxel (Sigma Aldrich) was measured and used as a standard (El-Maali *et al.*, 2018).

2.3.3. Fourier transforms infrared spectroscopy (FT-IR)

Spectroscopic investigations on paclitaxel samples are of importance in the present and studied extensively by many scientists (Schmitt *et al.*, 2015). The infrared spectrum of a compound is the superposition of the absorption bands of specific functional groups and can be used as a fingerprint for unknown compounds identification in comparison with recorded reference spectra. To detect the functional groups of paclitaxel in the fungal extract, powders of paclitaxel were tested by the FTIR method using a spectrophotometer model was analyzed using the KBr pressed disk technique (Thermo Scientific Nicolet iS10 FT-IR Spectrometer) at Chemistry Department, Faculty of Science, Assiut University.

2.3.4. MTT cell viability assay

Effect of methanol and chloroform extracted paclitaxel from fungal culture on MCF7 breast cancer cells viability and toxicity were compared to that of pure taxol were investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) MTT cell viability assay as previously described (Ismail *et al.*, 2013). In brief, about 1 x 10^4 cells/well were cultured in 96-well plates and then incubated for 24h. On the following day, different concentrations of extracted paclitaxel (methanol and chloroform) from the culture medium were added to the cells for 24h and positive controls (methanol and chloroform) were involved in the experimental design to avoid their cytotoxic effect on the cells. As well, pure taxol (purchases taxol) was applied to compare the cytotoxic activity of extracted paclitaxel and pure taxol. Next, fifty µl of MTT solution (2 mg/ml of MTT in PBS) were added to each well and left for 4 h, then the supernatants were removed carefully and 150 µl DMSO were added to each well. The 96-well plates were shaken for 10 minutes and then were read at A570 with a reference filter at A650 using an ELISA plate reader (Ismail *et al.*, 2013).

2.3.5. Chitosan extraction from fungal biomass

After fungal cultivation on sucrose malt medium, the culture (fungal biomass and medium) was used for paclitaxel production, then filtered by filter paper (Whatman No.1) and the fungal biomass residue was used for chitosan production. Dried fungal biomass was homogenized with 0.5 N sodium hydroxide solution (1:30, w/v) at 121˚C for 20 min for the deproteinization process. The alkali insoluble materials (AIM) were centrifuged at 10000 rpm for 15 min and subsequently washed with distilled water until the pH was completely neutralized, dried and weighted. Then, 1 gram of dried AIM was treated with 40 ml, 2% acetic acid at 95˚C for 6 h (Synowiecki and Al-Khateeb, 1997). The extract containing fungal chitosan was isolated by centrifugation at 10000 rpm for 15 min, the supernatant containing the chitosan was collected, adjusting pH to 10.0 with 2M NaOH followed by centrifugation at 10,000 rpm for 15 min for separation of chitosan. The precipitation was washed with distilled water until reaching pH 7, followed by 95% (v/v) ethanol (1:20, w/v). Finally, dried at 60˚C for 24 h. Fungal chitosan (virtually pure mycelial chitosan) yield was calculated by weight (g). The alkali-insoluble precipitate obtained after suitable alkali and acid extraction contained nearly pure chitosan (McGahren *et al.*, 1984). The crude chitosan yield of residual fungal biomass was calculated from the following equation (Abdel-Gawad *et al.*, 2017):

$$\text{Chitosan yield (\%)} = [\text{dry wt. of obtained chitosan} / \text{dry wt. of sample}] \times 100$$

2.4. Characterization of fungal Chitosan

2.4.1. Determination of glucosamine content

Fungal chitosan samples were hydrolyzed with 2 M hydrochloric acid at 110 ˚C for 2 h and the liberated D-glucosamine was determined by dinitrosalicylic acid according to Huet *et al.* (2006).

2.4.2. Infra-red spectroscopy

The structure of extracting mycelial chitosan was confirmed by infrared spectroscopy using a KBr pellet method in FTIR (Thermo Scientific Nicolet iS10 FT-IR Spectrometer) at Chemistry Department, Faculty of Science, Assiut University. FTIR spectra were recorded in the middle infrared (4000 cm^{-1} to 500 cm^{-1}).

2.4.3. Degree of deacetylation

The degree of deacetylation (DD) was determined according to the baseline method (Khan *et al.*, 2002). The IR spectrum recording procedure is the same as described above. According to the IR spectrum, the DD was calculated by measuring the absorbance ratio of A1655 and A3450 (Baxter *et al.*, 1992). The amide I band at 1655 cm^{-1} and the hydroxyl group absorption band at 3450 cm^{-1} were used as an internal reference, DD was assessed based on the following equation:

$$\text{Degree of deacetylation} = 100-[(A1655/A3450) \times 115]$$

2.4.4. Determination of antioxidant activity by reducing power measurement

The reducing power of chitosan extract was determined by the method of Chang *et al.*, (2002) with some modification. An aliquot of 0.5 ml chitosan sample (5:100 w/v), in 0.2% acetic acid solution was added to 0.1 ml of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 ˚C for 30 min, 0.1 ml of trichloroacetic acid (1% w/v) and 0.1 $FeCl_3$ (0.1% w/v) were added and left for 20 min. The result was recorded by measuring the amount of ferric ferrocyanide (Prussian blue) formed using spectrophotometer at 700 nm and ascorbic acid was used as a positive control.

3. Results

3.1. Morphological and molecular characterization of the fungus

The endophytic fungus isolated from avocado fruits was identified morphologically and genetically as *Neopestalotiopsis clavispora*. The isolate is characterized morphologically by dark brown, subglobose conidioma; conidia with 4 transverse septa, fusiform to clavate, smooth-walled, hyaline, 20-27 µm length (Judith-Hertz, 2016) as shown in Figure 1. Morphological features of the fungus were confirmed by 18S rRNA sequence analysis (Maharachchikumbura *et al.*, 2014). A partial 18S rRNA gene sequence of approximately 501 base pairs of fungal strain ASU1 has a sequence with 99 % similarity with *Neopestalotiopsis clavispora* (KR052094), *N. foedans* (KU593530) and *Pestalotiopsis paeoniicola* (EU400221) that is available in the Genbank database. A phylogenetic tree was constructed from multiple sequence alignment of 18S rRNA gene sequences (Figure 2). The nucleotide sequence of fungal strain ASU1 was deposited in Genbank with accession number KY624416.

Figure 1. Image of *Neopestalotiopsis clavispora* growth on PDA medium (A), Neopestalatiopsis conidia with 4 transverse septa, fusiform to clavate, and smooth-walled (B).

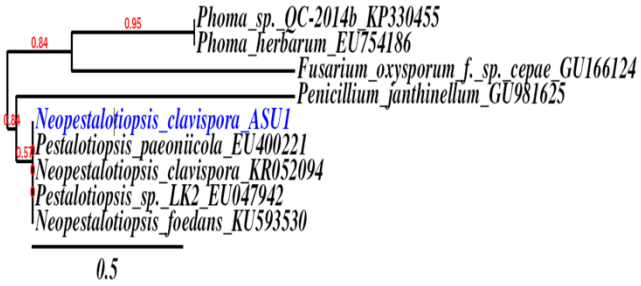


Figure 2. Phylogenetic tree of *Neopestalotiopsis clavispora* ASU1 (KY624416).

3.2. Production of paclitaxel

In the present study, *Neopestalotiopsis clavispora* KY624416 was grown on malt sucrose medium with a higher growth rate recording 14.2±0.095 g/l dry biomass with potentiality for anticancer compounds production. HPLC analysis of paclitaxel produced by endophytic fungus *Neopestalotiopsis clavispora* KY624416 elucidated that fungal strain could produce anticancer compounds in liquid medium recording a single peak identical with the retention time of authentic paclitaxel (Figure 3). Although a defined identical single peak was produced from HPLC

analysis, it may contain other taxanes (Figure 3). The quantitative analysis of paclitaxel and related taxanes was performed with LC/MS-MS that may be very important to discriminate paclitaxel from other closely related taxanes. In the present study, it was confirmed that the LC/MS chromatographic analyses are highly accurate showing that *Neopestalotiopsis clavispora* KY624416 paclitaxel extract, had four peaks at m/z from 876.7 to 877.7, that similar to the peak obtained with authentic paclitaxel (Figure 4). LC/Ms-Ms analysis of fungal paclitaxel confirmed that fungal strain could produce paclitaxel with concentration recording 100.6 µg/l.

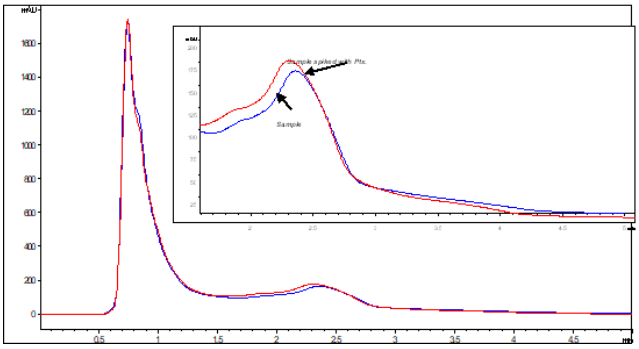


Figure 3. HPLC chromatogram of standard taxol and fungal paclitaxel isolated from *Neopestalotiopsis clavispora* ASU1 (KY624416).

3.3. FT-IR analysis

The resultant absorption spectrum indicates the presence of various chemical bonds and functional groups present in the sample. The Fourier transform infrared spectroscopy (FT-IR) analysis measures the selective absorption of light by the vibration modes of specific chemical bonds in the sample determining several functional groups such as OH-hydroxyl and amide (-NH) groups stretch (3392.65 cm^{-1}), aliphatic CH stretch (2900 cm^{-1}), aromatic ring C=C stretch was positioned at 1652.61 cm^{-1}, NH stretching (1455.93 cm^{-1}), COO stretching frequency was observed at 1245.11 cm^{-1} and aromatic C and H bend (1082.52 cm^{-1}) as shown in Figure 5.

3.4. Cell viability

Cell viability was investigated in MCF-7 breast cancer cell line after 24 h of treatment with different extracted (methanol, chloroform) paclitaxel concentrations (0.0, 0.01, 0.05, 0.1, 0.15, and 0.3 µM) and compared with purchases taxol using MTT assay. Quantitatively, it was shown that taxol drug exerted a dose-dependent cytotoxic effect. Statistically, the significant cytotoxic effect was ($P< 0.05$) at 10, 50 and 100 nM, ($P< 0.01$) at 150 nM and ($P< 0.001$) at 300 nM while the extracted fungal paclitaxel exerted a significant cytotoxic effect ($P< 0.05$) only at 300 nM. Regarding the comparison among fungal taxol, our results revealed that the methanol extract was the highly effective compared to the other tested extracts which exerted more cytotoxicity at nearly all concentrations. Statistically, the differences among fungal taxol showed no significant cytotoxic effect on MCF-7 breast cancer cells (Table 1).

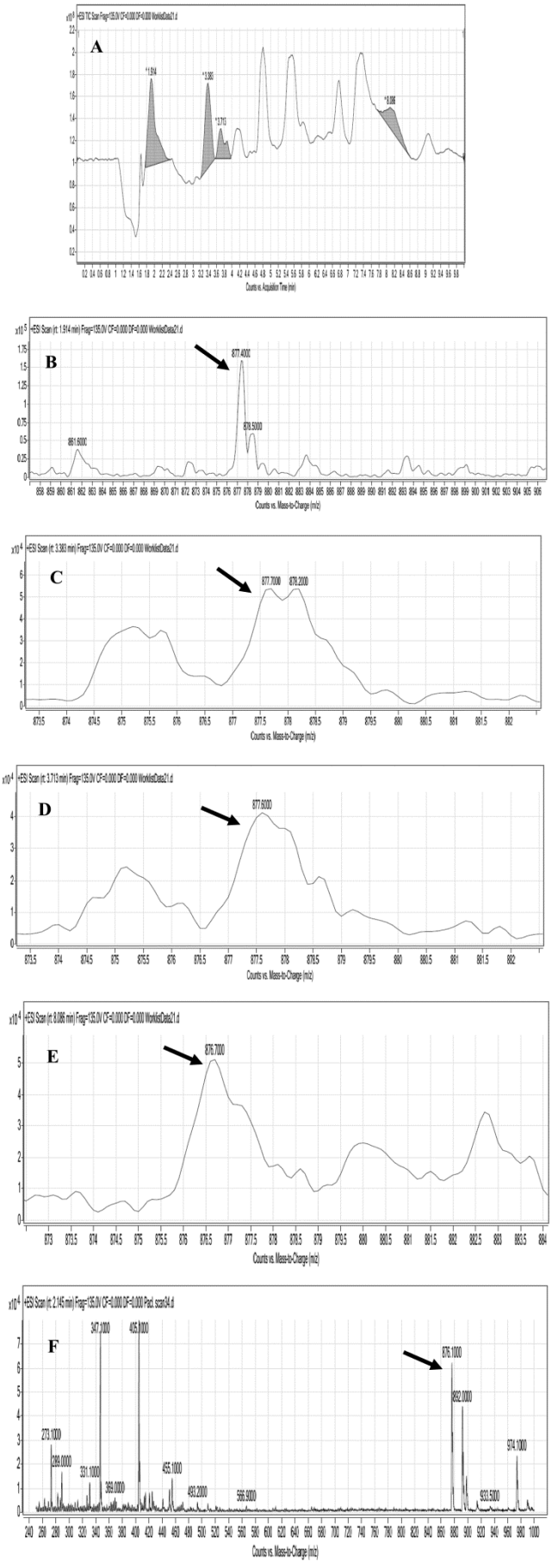


Figure. 4. LC/Ms chromatograms showing fungal paclitaxel scan (A) with four peaks at m/z from 876.7 to 877.7 (B-F), that similar to the peak obtained with authentic paclitaxel (E).

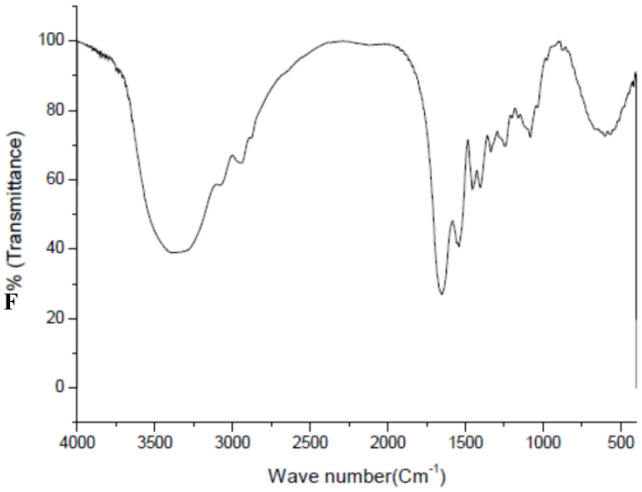


Figure 5. FT-IR spectrum of paclitaxel produced by *N eopestalotiopsis clavispora* ASU1.

3.5. Chitosan production from fungal biomass wastes

The results obtained showed that the chitosan yield from fungal biomass represented 5.94% of fungal dry biomass glucosamine content of extracted fungal chitosan was estimated by 3, 5 dinitrosalysilic acid reagent yielding 73.53 % of extracted chitosan (Figure 6).

3.5.1. Characterization of fungal chitosan

3.5.1.1. FT-IR analysis

Chitosan extracted from residual fungal biomass after the fermentation process for paclitaxel production was subjected to FT-IR analysis. The FT-IR spectra in Figure 7 showed typical absorption bands of chitosan: the OH stretching band at 3427.6 cm^{-1} the stretching vibrations overlapped with N-H stretching band; the C-H band within 2924.51 cm^{-1}. The band identified as an amine group (N-H bending bands) absorbed infrared between 1651.4 cm^{-1} - 1566.7 cm^{-1}. The skeletal vibrations involving the C-O-C stretching band at 1035.57; the -CH_2 bending centered at 1416.49 cm^{-1}. Also, the spectra showed the amide bonds at approximately 1651.40 and 11566.73 cm^{-1}; amide I band at 1651.4 and amide II band at 1566.73 cm^{-1}. The IR spectroscopic method is used to determine the degree of deacetylation (DD) value of mycelial chitosan. The DD value of mycelial chitosan obtained from submerged fermentation of *Neopestalotiopsis clavispora* was 54.60% (Figure 6).

Table 1: Mean values ± SD of absorbance (MTT assay) and cell viability percentage in breast cancer cell line (MCF7) after 24 hr of different doses of different extracted taxol treatment

Treatment	Standard taxol (purchases taxol)		Fungal paclitaxel			
			Methanol extract		Chloroform extract	
Dose	Mean±SD	Viability%	Mean±SD	Viability%	Mean±SD	Viability%
0 nM	0.76±0.07	100	0.56±0.03	100	0.50±0.007	100
10 nM	0.59±0.05[a]	77.2	0.51±0.1	87.9	0.44±0.056	87.3
50 nM	0.51±0.06[a]	66.5	0.49±0.02	85.2	0.42±0.066	83.4
100 nM	0.45±0.03[a]	58.2	0.48±0.1	81.9	0.41±0.038	80.6
150 nM	0.33±0.05[b]	43.3	0.46±0.12	78.1	0.40±0.039	78.6
300 nM	0.2±0.03[bc]	26.7	0.44±0.11[a]	74.5	0.38±0.020[a]	75.5

3.5.2. Antioxidant activity of chitosan

The antioxidant activity of chitosan extracted from *Neopestalatiopsis clavispora* waste biomass was studied using ferric reducing antioxidant activity recording 0.263±0.051 mg/ml, this result revealed that fungal chitosan has potent reducing power and antioxidant power.

4. Discussion

4.1. Production of paclitaxel

Currently, most of the paclitaxel produced for medical uses is synthesized by a chemical semi-synthetic approach (Holton *et al.*, 1995). However, the extraction of taxol from Taxus plants is relatively complex with low yields, depending on epigenetic, seasonal and environmental conditions (Jiang *et al.*, 2021; Zhu and Chen 2019) . In the eighteenth century, endophytic fungi become a useful tool for the paclitaxel production process of the future, great progress in this field have been obtained. However, despite its many advantages as, shorter generation time, higher growth rate, easily genetic manipulation, low substrate costs for endophytic fungi growth and easy recovery of paclitaxel from the fermentation medium; some impediments are resulting from yields and titers remain low compared with that of the taxes trees (Demain and Solomon, 1981; Stierle *et al.*, 1993).

In the present study, the endophytic fungus isolated from avocado fruits was identified based on morphological and genetic analysis as *Neopestalotiopsis clavispora* and was deposited in Genbank with accession number KY624416. *N. clavispora* KY624416 showed a high potentiality for paclitaxel production, an anticancer compound which was analyzed by HPLC and LC/MS-MS analysis recording 100.6 µg/l. In this respect, *Pestalotiopsis microspora* isolated from the inner bark of Himalayan yew could produce taxol in mycelial culture (Strobel *et al.*, 1996b). Paclitaxel was originally produced from the Taxus plant, it was reported that the gene responsible for paclitaxel production may transfer from the plant to corresponding endophytic fungi (Stierle *et al.*, 1993). These fungi may produce paclitaxel for competition and survive inside their hosts (Young *et al.*, 1992). On the other hand, paclitaxel biosynthetic gene candidates in endophytic fungus *Penicillium aurantiogriseum* NRRL 62431 are quite different from those in hosts and they provided evidence that horizontal gene transfer is unlikely to have occurred. Several

endophytic fungi from different host plants have been investigated for their ability for paclitaxel production (Mirjalili *et al.*, 2012; Yang *et al.*, 2014). In our study we observed paclitaxel production by endophytic fungus *Neopestalotiopsis clavispora* KY624416 during this batch fermentation process is quite good in comparison with various endophytic fungi reported in the literature.

FT-IR analysis for extracted paclitaxel sample showed absorption spectrum and functional groups such as OH-hydroxyl and amide (-NH) groups stretch, aliphatic CH stretch, aromatic ring C=C stretch, NH stretching, COO stretching frequency and aromatic C and H bends. The observation of the vibration spectrum of paclitaxel in comparison with standard taxol spectrum (Raj *et al.*, 2015; Vennila and Muthumary, 2011). Consequently, FT-IR spectroscopy was widely used recently by researchers to verify the chemical characteristics of paclitaxel in comparison with standard available spectrum (Hiremath *et al.*, 2013; Kathiravan *et al.*, 2014; Schmitt *et al.*, 2015).

4.2. Cell viability

From cell viability results using MCF-7 breast cancer cell line, it was found that the most effective one of paclitaxel extracts is the methanol extract which exerted more cytotoxicity at nearly all concentrations. As well, the IC50 values of strong cytotoxic activity of commercial taxol, methanol extract and chloroform extract were found against MCF-7 breast cancer cell line at 158.1 nM, 621.7 nM, 667.3 nM and 596.4 nM, respectively. As well, fungal paclitaxel is a proven anti-cancer agent that has been tested in various human carcinoma cell lines, such as MCF-7 (Kumaran *et al.*, 2012; Pandi *et al.*, 2011), BT220 (Gangadevi and Muthumary, 2007; Kumaran *et al.*, 2012), HL251 (Kumaran *et al.*, 2012), Hep G2 cell lines (Rajendran *et al.*, 2013). Supporting the earlier findings that at low concentration taxol could inhibit cell proliferation by blocking mitosis (Gangadevi and Muthumary, 2007; Kumaran *et al.*, 2012). Also, *Cladosporium oxysporum* taxol showed cytotoxic activity towards HCT 15 cancer cell line for 24 h treatment with an IC50 value of 3.5 µM concentration (Raj *et al.*, 2015). In conclusion, fungal endophytes are gaining importance because of their potentiality to produce medically important bioactive compounds.

4.3. Chitosan production from fungal biomass residue

The chitosan yield extracted from fungal biomass wastes represented 5.94% of fungal dry biomass and the glucosamine content of extracted fungal chitosan was 73.53 % of extracted chitosan (Figure 6). Chitosan yield by endophytic fungus *Neopestalotiopsis clavispora* KY624416 grown on sucrose malt broth medium is quite good when compared with values using various fungi using different substrates reported in the literature in this instance. Chitosan was extracted from different fungal species such as, *Cunninghamella elegans* (0.75 g/l), *C. blakesleeana* (9.4 % of fungal biomass), *Rhizomucor miehei* (13.67 %), *Mucor rouxii* (1.2 %) *Cladosporium Cladosporioides* (25.2 mg/g) with degree of deacetylation 81 %, 35 %, 80.6 % and 59 %, respectively (George *et al.*, 2011; Miyoshi *et al.*, 1992; Tajdini *et al.*, 2010; Tayel *et al.*, 2016). As well as the extracted chitosan of some fungi was tested for their biotechnological applications in water pollutant elimination (heavy metals and waterborne microorganisms) and removal of toxic pollutants biocontrol of postharvest diseases (Betchem, *et al.*, 2019; Cortés-Rivera *et al.*, 2021)). Furthermore, these results provide a promising approach for concomitant production of paclitaxel and chitosan sources and increase the possibility for production and extraction of fungal chitosan with well-defined properties at an industrial scale.

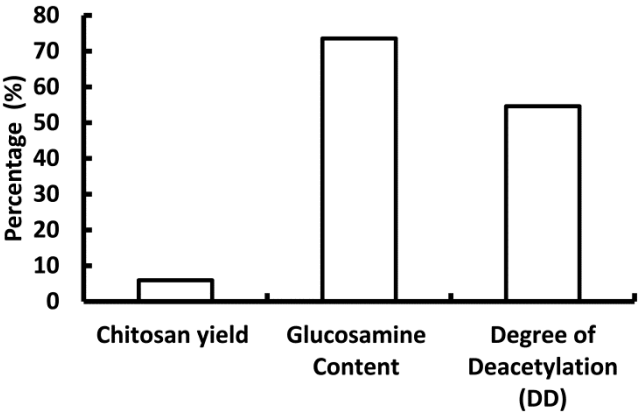


Figure 6. Percent of chitosan yield from fungal biomass residue after paclitaxel extraction, glucosamine content in extracted chitosan and the degree of deacetylation.

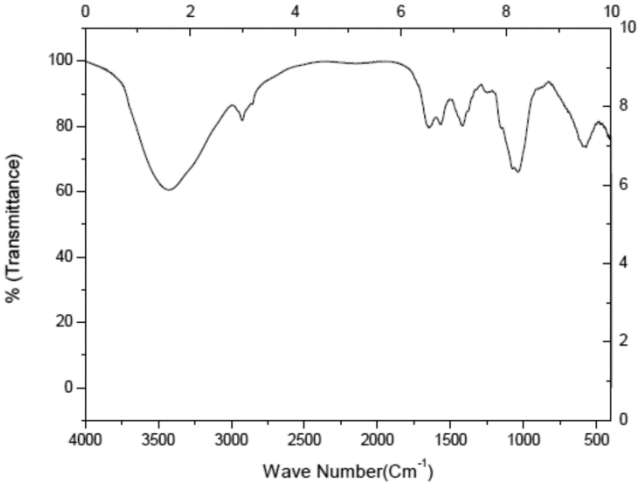


Figure 7. FT-IR spectrum of chitosan extracted from fungal biomass.

IR spectroscopy of chitosan extracted from residual fungal biomass provided a relatively quick, simple method used for the qualitative and quantitative determination of chitin and chitosan characteristics, mainly specific absorption groups present in the recovered product, the degree of acetylation/deacetylation and chitosan impurities (Baxter *et al.*, 1992). The FT-IR spectra showed absorption bands; the OH stretching band, the C-H band, an amine group (N-H bending bands) as recorded by Pavia *et al.*, (2014). As well as, the skeletal vibrations include the C-O-C stretching band; the -CH_2 bending (Kaya *et al.*, 2015; Kumirska *et al.*, 2010; Negrea *et al.*, 2015). Also, the spectra showed the amide bonds; amide I band at 1651.4 and amide II band.

The current results were in harmony with the findings of Kasaai *et al.*, (2000); Khan *et al.*, (2002); Also, FT-IR analysis of chitosan obtained from the mycelia of Basidiomycetes exhibited several bands similar with internal reference spectra of chitosan such as amide bonds at approximately 1655 and 1313 cm^{-1} (Di Mario *et al.*, 2008). In the current study, other functional groups included hydroxyl stretching band at 3427 cm^{-1}, amide I band at 1655, primary amine band at 1638 - 1561 cm^{-1} amide II band at 1561 cm^{-1} and amide III bands at 1320 cm^{-1}. These absorption bands preliminary approved the fungal extracted product to be chitosan.

The IR spectroscopic method is used to determine the degree of deacetylation (DD) value of mycelial chitosan. The degree of deacetylation of chitosan extracted from *Neopestalotiopsis clavispora* was 54.60% which is considered as one of the most important chemical characteristics influencing solubility, chemical reactivity and biodegradability, which in accordance influence the performance of chitosan in many applications (Abdel-Salam, 2013; Morin-Crini *et al.*, 2019) . Interestingly, these desirable physicochemical properties of fungal chitosan could increase the potential application for food, cosmetic, chemical and pharmaceutical industries. Consequently, fungal chitosan fermentative production is considered a good alternative to minimize environmental pollution from traditional chitosan production by strong alkali, furthermore increase the feasibility of a new, simple and green technology for the production of value-added chitosan (Dhillon *et al.*, 2013a).

4.4. Antioxidant activity of chitosan

The antioxidant activity of chitosan produced from *Neopestalatiopsis clavispora* was evaluated using ferric reducing antioxidant activity (Zimoch-Korzycka *et al.*, 2016). Hydroxyl radicals display a small diffusion capacity and high reactivity in the generation of cellular damages. These injuries speed up the aging process causing cancer and numerous diseases (Kehrer and Klotz, 2015). So, removing OH radicals is important for antioxidant defense in cell and food systems. Reducing power measures the ability of a sample to act as an electron donor and therefore, reacts with free radicals converting them to more stable products and thereby terminate radical chain reaction (Park *et al.*, 2004). Fungal chitosan reducing power activitiy was 0.263±0.051 mg/ml, this result revealed that fungal chitosan has potent reducing power and its antioxidant power may be attributed to hydroxyl groups (OH), and amine groups.

5. Conclusion

Recently, the production of paclitaxel by endophytic fungi has gained much attention due to the need for cost-competitive and alternative sources for taxol production from *Taxus* plants. The present paper provides a promising approach through integrating a system for paclitaxel production from malt sucrose medium by endophytic fungus *Neopestalotiopsis clavispora* with chitosan production from residual fungal mycelium wastes. Paclitaxel productivity by endophytic fungus *Neopestalotiopsis clavispora* (100.6 µg/l) with a significant cytotoxic effect on MCF7 cell line revealed the possibility and potentiality of this fungal strain for paclitaxel industrial biotechnological processes. Furthermore, the available fungal mycelium wastes, resulting from the paclitaxel extraction process, represent a promising candidate for chitosan production as compared to traditional sources from crustacean shells. The remarkable findings of this study have been proven to be practically important in the development of paclitaxel production processes and enhancing low-cost chitosan production from the utilization of fungal mycelium wastes. Presently, the industries sustain losses for the safe disposal of wastes, which leads to an increase in the final production cost of the products so, this paper is expected to provide a competitive economic outcome for industrial concomitant of paclitaxel and chitosan production by endophytic fungi.

6. Conflict of interest

The authors declare that there is no conflict of interest.

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