

# Investigation of Secondary Metabolites and its Bioactivity from *Sarocladium kiliense* SDA20 Using Shrimp Shell Wastes

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## ABSTRACT

**Introduction:** In this study we isolated bioactive compounds using different chromatographic techniques from culture filtrate of *Sarocladium kiliense* SDA20 grown in fermentation media containing shrimp shell waste as substrate under optimum conditions. Antibacterial and antibiofilm activities of crude extract and purified compounds were evaluated. **Methods:** The test fungi strain *Sarocladium kiliense* SDA20 was isolated from Egyptian soil and identified by 18 S ribosomal RNA. Optimization conditions were carried out in fermentation media containing shrimp shell waste as sole carbon source, inoculated by 10<sup>6</sup> spores/ml of *Sarocladium kiliense* SDA20 at pH 7.0 produce 84.5% of the total toxins. Different chromatographic techniques for ethyl acetate extract of culture filtrate of fungi were used resulting in isolation of pure compounds were elucidated spectroscopically and comparing their data in literature. GC/MS analysis of extract was used for identification of other chemical compounds. Antibacterial and biofilm activity was evaluated using MTT assay. **Results:** Five compounds for the first time were identified: Cholest-5-en-3-ol (C1), Palmitic acid (C2), Oleic acid (C3), Nicotinamide (C4), Tricin (C5). GC-MS analysis showed the presence of twenty-seven compounds. Antibacterial activity of crude and pure compounds displayed a strong inhibitory activity against *Bacillus subtilis*. C1, C4, C5 showed moderate activity against *Escherichia coli*, followed by C2, C3. Meanwhile, *Staphylococcus aureus* was less susceptible to pure compounds with low activity of C3, C5. The crude extract and pure compounds displayed biofilm inhibition activity against four pathogenic bacterial strains. **Conclusion:** Shrimp shell wastes are one of the economic bioproducts used for the production of bioactive secondary metabolites from fungi.

**Key words:** Antibacterial activity, Antibiofilm activity, GC/MS analysis, *Sarocladium kiliense* SDA20, Secondary metabolites, Shrimp waste shell.

## INTRODUCTION

Utilization of shrimp waste an alternative technology to replace dangerous chemical methods that addresses the future trends in total utilization of shrimp waste for recapture of bioactive compounds. Low molecular weight fungal secondary metabolites produced by a wide variety of fungi. These compounds determined as mycotoxins.<sup>1,2</sup> Mycotoxins are a varied group of compounds produced by an extensive range of different fungi, normally after a period of balanced growth.<sup>3</sup> Fungi are capable to produce one or more mycotoxin. Therefore, several mycotoxins are often at the same time found in a single product.<sup>4</sup> Many organisms produce large numbers of secondary metabolites and the complexity and diversity is sometimes amazing. Secondary metabolites are organic compounds can be used in traditional medicine and in modern times, as pharmaceuticals, flavoring in foods, fragrances in cosmetics, agrochemicals and drinks, etc.<sup>5,6</sup> Filamentous fungi can be cultivated beneath in fermentation medium contain nutrients for fungal growth, changing in this medium composition affects the biomass yield and quality as well as metabolites.<sup>7</sup> The high cost of synthetic media has interested to use another new fermentation media designed from inexpensive by-products and wastes, which are able to support

and fulfill nutritional requirements for microbial growth.<sup>8</sup> Substrates used for cultivation of fungi must be easily biodegradable, easily available, cheap, and contain sufficient required micro and macro-nutrients.<sup>9</sup> Shrimp aquaculture is an attractive economic activity of great impact and commercial importance.<sup>10</sup> The waste generated from the worldwide production and processing of shell-fish and fish scales is a serious problem of growing magnitude. This abundant waste may pose environmental hazard due to the easy deterioration. The use of shrimp shell waste due to their low commercial value and applications of these residues can be used as good substrate by fungi for production of secondary metabolites from cheap and low-cost substrate.<sup>11</sup> The waste created from the overall generation and preparing of shellfish and fish scales is difficult issue of developing greatness. Recently, bacterial biofilm considered as one of the major public health problems. This process gives bacteria the ability to maintain their attachment to living and non-living surface and to keep the bacterial cells together by production of a complex exopolymers formed from proteins, polysaccharides and nucleic acids.<sup>12-14</sup> Several reports have now clearly confirmed that, biofilm play a significant role in the persistence of pathogenic bacteria.<sup>15</sup> Biofilms are considered as a source of pathogenic bacteria

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that are involved in many infectious diseases.<sup>16,17</sup> Despite several attempts to find drugs that specifically target bacterial biofilms, still there is undeniable need to discover anti-biofilm agents that could inhibit bacterial biofilm formation. The aim of this study was isolation of bioactive compounds using different chromatographic techniques from culture filtrate of *Sarocladium kiliense* SDA20 accession number (MN636431) grown in fermentation media containing shrimp shell waste as substrate under optimum conditions. Then evaluation the biological activities (antibacterial activity and antibiofilm activity) of the crude extract and the purified compounds.

## MATERIALS AND METHODS

### Microorganisms

The test fungal strain *Sarocladium kiliense* SDA20 was isolated from the Egyptian soil at El Behira province and identified by 18 S ribosomal RNA. Cultures were kept on potato dextrose slants and kept at 4 °C.

### Isolation media

Czapek-Dox agar (Haung and Ling, 1973) g/l: NaNO<sub>3</sub> 2.0; K<sub>2</sub>HPO<sub>4</sub> 1.0; KCl 0.5, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5; FeSO<sub>4</sub> 0.001; sucrose 30; agar 20 and 1000 ml distilled water, pH 6.5 – 7.0.

### Phylogenetic identification of fungal isolates

#### DNA extraction

The molecular identification of the selected fungal strain was carried out by sequencing of the 18srRNA gene. The extraction of the genomic DNA was performed using Qiagen DNeasy Mini Kit following the manufacturer's manual.

#### PCR amplification

The PCR reaction mixture was as follows: (1 µg genomic DNA, 1 µL (20 uM of each primer), 10 mM dNTPs mixture, 2 units of Taq DNA polymerase enzyme and 10 µL 5X reaction buffer). The amplification reactions were performed using 6 primers; 6 primers ITS1 (5' TCCGTAGGTGAACCT

GCG-3')/ ITS4(5' -TCCTCCGCTTATTGATATGC-3') the following PCR thermal profiles: denaturation step at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 90 sec, and a final extension step of 72 °C for 5 min.

#### Sequencing

The amplified products were examined by electrophoresis and sequenced in Macrogen Companies, South Korea. The sequence produced was analyzed by using BLASTN program, to study the similarity and homology of the 18S rRNA gene sequences with the similar existing sequences available at NCBI database.

#### Substrate used

Shrimp shell waste was collected, washed and dried in oven at 70°C, then grinded, stored until used.

#### Fermentation media

Fermentation was carried out in 250 ml Erlenmeyer flask each containing 50 ml of fermentation medium consist of (g/l): shrimp shell waste powder 20, NaNO<sub>3</sub> 2.0, K<sub>2</sub>HPO<sub>4</sub> 1.0, KCl 0.5 gm, MgSO 4.7, H<sub>2</sub>O 0.5, FeSO<sub>4</sub> 0.001- and 1000-ml distilled water, pH 6.5 - 7.0. One ml of 10<sup>6</sup> spore suspension of each fungal strain were inoculated in each flask and incubated at 28±30 °C for 7 days at 180 rpm. (shrimp shell waste used as carbon source).

## Optimization conditions

### Effect of different inoculum size on metabolites production

Different inoculum size ranged from (10<sup>4</sup>, 10<sup>6</sup>, 10<sup>8</sup> & 10<sup>10</sup>) spores/ml were examined to determine total secondary metabolites production.

### Effect of different initial pH values on metabolites production

Different initial pH values of fermentation media for compounds production were adjusted at (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 & 8.0) using dilute HCl or NaOH solutions. Inoculated medium was then incubated for 7 days.

### Instruments

NMR spectra were recorded on a Jeol Ex-500 spectrometer: 500 MHz (<sup>1</sup>H-NMR), 125 MHz (<sup>13</sup>C-NMR). UV spectro photometer (Shimadzu UV-240). ESI-MS were recorded on a Waters-Micromass Quattro Premier Triple Quadrupole mass spectrometer. Column chromatography (CC) was carried out on silica gel F254 (Merck) in glass blades. TLC was performed with silica gel 60 GF254 plates (Merck, Darmstadt, Germany), then the plates were visualized using UV light and by spraying with vanillin in H<sub>2</sub>SO<sub>4</sub>. The GC-MS was performed using the GC-MS system (Agilent Technologies), equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A). The GC was equipped with HP-5MS column (30 m x 0.25mm internal diameter and 0.25 µm film thickness).

### Preparing extract

The culture filtrate (700 g) of *Sarocladium kiliense* SDA20 was extracted with ethyl acetate till exhaustion. The extract was evaporated under reduced pressure to yield (30 g) of crude extract. A portion of this extract was subjected to column chromatography for compounds isolation, part was kept for GC-MS analysis, and other was stored for biological study.

### Isolation of the compounds

The crude extract was subjected to silica gel column chromatography, eluted with petroleum ether and ethyl acetate step-gradient, starting with 100% petroleum ether with increasing polarity, in the ratio (100:0, 9:1, 8:2, 7:3, 6:4, 4:6, 3:7, 2:8, 1:9, 0:100). The elutes were monitored using TLC, and were viewed under UV light (254 and 365 nm) and by spraying with 1% vanillin/ 5% H<sub>2</sub>SO<sub>4</sub>/ EtOH reagent, followed by heating at 100°C. The fractions obtained were collected and combined based on TLC profiles, to give five major fractions from which 5 compounds were obtained. Five different fractions were eluted by different concentrations of ethyl acetate in petroleum ether (Table 1) and purified by TLC on silica gel "G" plate using petroleum ether/ ethyl acetate (9:1) as developing system, giving compounds C 1, C 2, C 5, and (8:2) developing system giving compounds C 3, C 4. The compounds were identified with their physical and spectroscopic data and comparing data with reported literature.

### Gas chromatography–mass spectrometry analysis (GC-MS)

The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at

**Table 1: Different ethyl acetate concentrations.**

Fractions no.	Different ethyl acetate concentrations %
1	10
2	20
3	30
4	70
5	80

Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was equipped with HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 µm film thickness). Analysis was carried out using Helium as the carrier gas at a flow rate of 1 ml/min at a split-less mode, injection volume of 1 µl and the following temperature program: 60°C for 2 min; rising at 5°C/min to 300°C and held for 10 min. The injector and detector were held at 280°C and 300°C, respectively. Mass spectra were obtained by electron ionization voltage (EI) at 70 eV and using a spectral range of m/z 50-550 and solvent delay 3 min. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

### Antibacterial activity

To measure the antibacterial activity of the crude and pure compounds. Gram-positive bacteria (*Staphylococcus aureus* ATCC 9144 and *Bacillus subtilis* ATCC 29212) and gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) were used as test organisms and antibacterial tests were performed as in.<sup>18</sup> The tests were performed in 96-well flat polystyrene plates. 10 µl of test extracts (final concentration of 250 µg/ml) were added to 80 µl of lysogeny broth (LB broth) followed by addition of 10 µl of bacterial culture suspension (log phase), then the plates were incubated overnight at 37°C. After incubation, the positive antibacterial effect of the tested compound observed as clearance in the wells, while compounds that didn't have an effect on the bacteria, the growth media appeared opaque in wells. The absorbance was measured after about 20 h at OD600 in a Spectrostar Nano Microplate Reader (BMG LABTECH GmbH, Allmendgrun, Germany). The positive control (Bacteria plus distilled water), while, negative control (growth media plus distilled water).

### Minimum inhibitory concentration (MIC)

To determine the MIC of the tested compounds that displayed the antibacterial activity, 80 µL of lysogeny broth (LB broth) were mixed with 10 µL of different concentrations of test compounds and followed by addition of 10 µL of a fresh culture (1×10<sup>6</sup> CFU/mL) of test microorganisms. Micro plates were incubated at 37°C for 24 h. MIC are defined as the lowest concentration of the antimicrobial agent that inhibited the growth of microorganisms.<sup>19</sup>

### Antibiofilm activity

The biofilm inhibitory activity of the ethyl acetate crude extract and pure compounds were measured using 96-well flat polystyrene plates toward and four clinical microbes comprising gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) according to method of<sup>20</sup> with some modifications. Briefly, each well was filled with 180 µL lysogeny broth (LB broth) then inoculated with 10 µL of pathogenic bacteria followed by addition of 10 µL of samples along with control (without test sample). The plates incubated at 37 °C for 24 h and after incubation, content in the wells were removed and wells washed with 200 µL of phosphate buffer saline (PBS) pH 7.2 to remove free floating bacteria and left to dry at sterilized laminar flow for 1hr. For staining, 200 µL/per well of crystal violet (0.1%, w/v) were added for 1hr then excessive stain removed and plates kept for drying. Further, dried plates were washed with 95% ethanol and optical density was determined at optical density 570 nm by using a Spectrostar Nano Microplate Reader (BMG LABTECH GmbH, Allmendgrun, Germany).

## RESULTS

### Collection of samples and isolation of fungal isolate

Soil samples were collected from El Saf Province -Giza Governorate . Soil was taken at 10 cm depth. Soil samples were firstly sieved and then

dried for 5 days at 25°C. The samples were kept after drying at 10 °C followed by fungal strain isolation. Serial dilution of soil suspension was prepared up to 10<sup>-6</sup> dilution. Then, 0.1mL of suspension from dilutions 10<sup>-3</sup> to 10<sup>-6</sup> was spread on Potato dextrose agar media plates at 28 ± 2 °C for 6–8 days until the fungal growth colonies was observed. The fungi isolated on culture medium from soil were further purified by using streak method. Routinely, the purified fungal cultures were transferred by streaking every 6 to 8 days onto fresh Potato dextrose agar plates. Kept in refrigerator at 4°C.

### Phylogenetic identification of fungal isolates

The fungal isolate *Sarocladium kiliense* SDA20 was identified by sequencing of its ITS region. The obtained sequence was aligned with other identified strains in the Gene bank database

using online BLAST tool to determine the similarity score (<http://www.blast.ncbi.nlm.nih.gov/Blast>). The obtained result confirmed a very close similarity of the gene sequence with 97.45% homology of the isolate SDA20 with *Sarocladium kiliense* strain. The isolates identified as *Sarocladium kiliense* SDA20 and submitted in the gene bank under accession number MN636431 The phylogenetic tree was constructed using MEGA X program<sup>21</sup> and neighbour-joining method<sup>22</sup> (Figure 1).

### Different inoculum size

The data recorded in (Table 2) showed that increase in inoculums size of *S.arocladium kiliense* MN636431strain led to increase in the total secondary metabolites required which produce its maximum percentage at 10<sup>6</sup> spores/ml. This results were obese to<sup>23</sup> who found that 9 × 10<sup>4</sup> spores /ml of *A. flavus* were suitable for metabolites produced from fish wastes. The fungal counts recorded for metabolites production in the shrimps are considered very high ranging from 1.7 x 10<sup>4</sup> spores /ml.<sup>24</sup>

### Different pH values

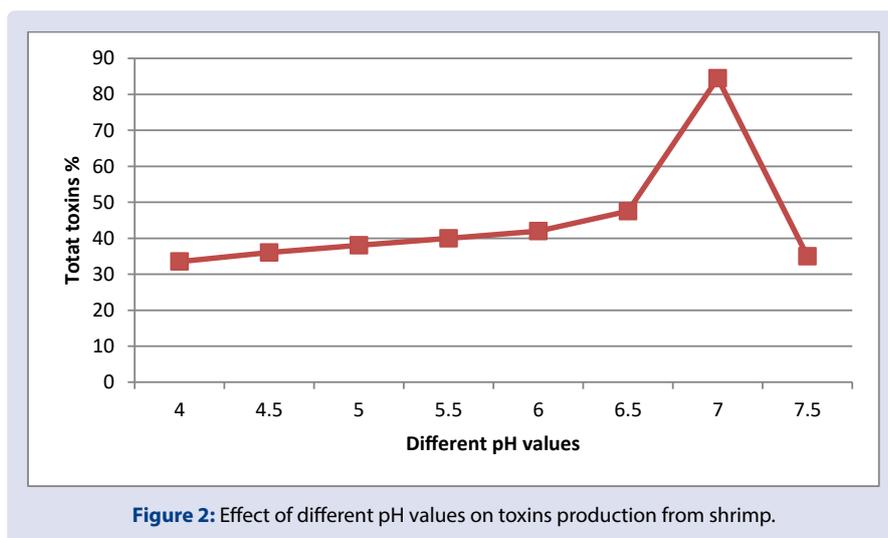
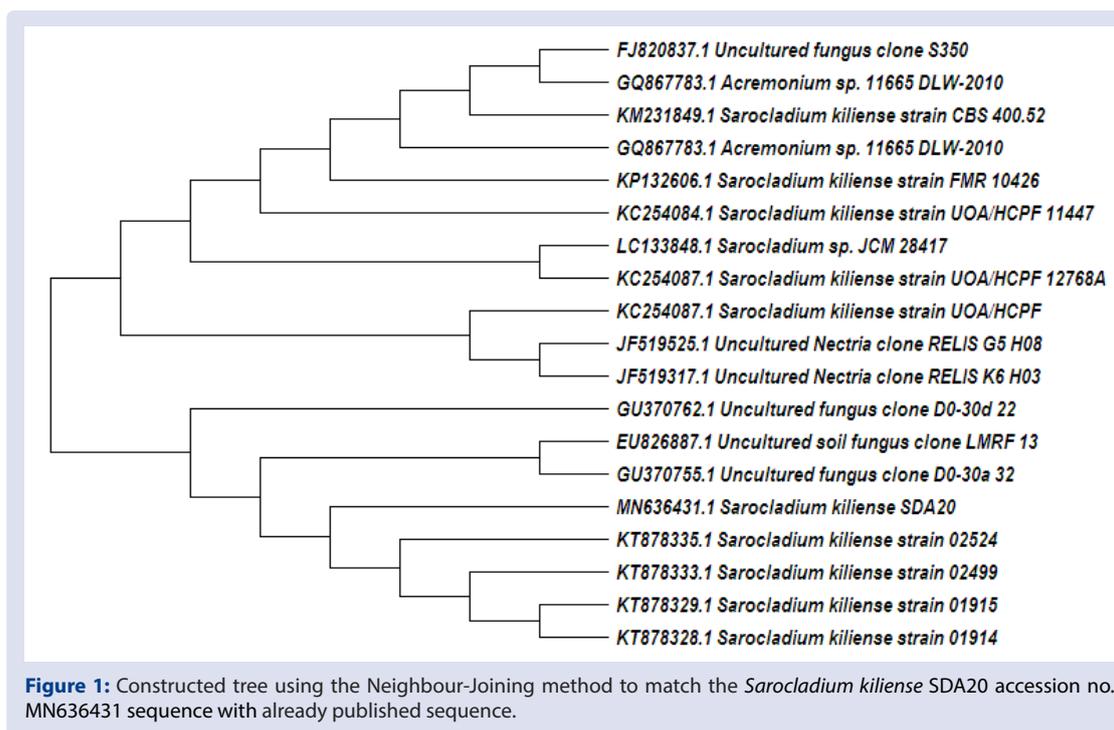
The results recorded in (Figure 2) revealed that increasing pH values led to increase percentage of total secondary metabolites production till reached 84.5 % at pH 7.0 then activity decreased. This results was coincided with<sup>25</sup> who found that the pH values of fermentation media for marine fungi between 6 and 7.5. The pH must be effected by the varieties of carbon and nitrogen sources also<sup>26</sup> found that the optimum pH of *T. thioerducens* was at pH 7.0 while *T. celericrescens* was optimum at pH 5.6–8.3, while this results in contrast with the first obligate *T. archae* cultured illustrated by<sup>27</sup> had a pH optimum of 4.2–4.8.

### Elucidation of the compounds

Five compounds from the ethyl acetate extract of culture filtrate of *S. kiliense* SDA20 were isolated for the first time as: **C1**: Cholest-5-en-3-ol, **C2**:Palmitic acid **C3**:Oleic acid, **C4**:Nicotinamide, **C5**:Tricin (Fig 3).The following compounds were isolated using different chromatographic techniques and identified by spectroscopic methods and comparing data with reported literature (Figure 3).

**Cholest-5-en-3-ol (C 1):** [M]<sup>+</sup> m/z 385, molecular formula C<sub>27</sub>H<sub>46</sub>O. <sup>1</sup>H NMR [500 MHz, CDCl<sub>3</sub>]: δ 5.36, 3.54 [m, H-6,3] respectively, δ 1.25-2.35 (m, H), 0.99, 0.69, 0.89, 0.84, 0.86 [Me-19,18,21,26,27]. <sup>13</sup>C NMR [125 MHz; CDCl<sub>3</sub>]: δ 37.2, 31.6, 71.8, 45.8, 140.7 [C (1, 2, 3, 4, 5)] respectively, 121.74, 29.22, 31.90, 50.22, 36.91 [C (6, 7, 8, 9, 10)] respectively, 21.08, 39.77, 42.28, 56.76, 24.31 [C(11,12,13,14,15)] respectively, 26.03, 56.04, 11.087, 19.03, 36.15 [C(16,17,18,19,20)] respectively, 18.78, 34.84, 27.26, 29.05, 26.41, 21.01, 21.02 [C (21,22,23,24,25,26,27)] respectively. Compound was confirmed by comparing its spectral data with the literature.<sup>28</sup>

**Palmitic acid (C 2):** Colorless powder, [M]<sup>+</sup> m/z 256. Molecular formula C<sub>16</sub>H<sub>32</sub>O<sub>2</sub>. UV: λ max 220 nm. <sup>1</sup>H NMR [CDCl<sub>3</sub>;500 MHz] δ [ppm]: 0.87 [t, J = 6.5 Hz. H<sub>3</sub>:16], 1.62 (2H, J = 7.5 Hz, 2H:3), 2.21



**Table 2: Effect of different inoculum size on toxins production from shrimp.**

Different inoculum size	Total toxins %
10 <sup>4</sup>	28.8
10 <sup>6</sup>	36.0
10 <sup>8</sup>	27.6
10 <sup>10</sup>	19.1

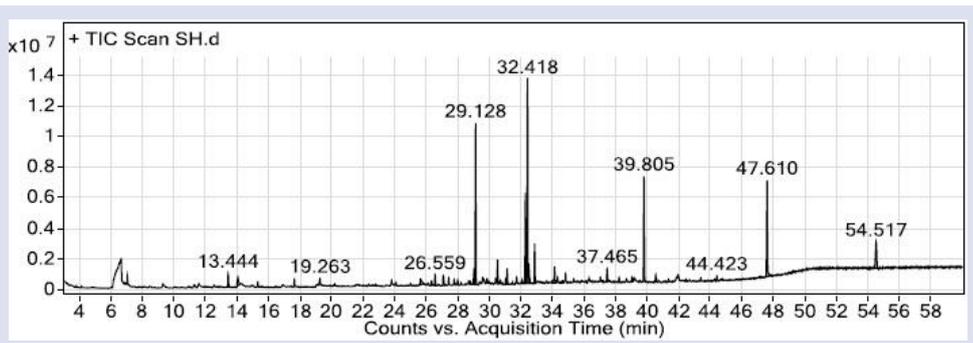
[t,  $J = 6.7$  Hz, 2H:2], 1.22-1.28 (24H, 2H<sub>4</sub>→2H<sub>15</sub>). <sup>13</sup>CNMR [CDCl<sub>3</sub>, 125MHz]  $\delta$  [ppm]: 14.4, 24.7, 31.8 [C (1, 2, 3)] respectively, 29.2 [m, C(4 -13)], 25.86, 34.2, 179.2 [C (14, 15, 16)] respectively. Compound was confirmed by comparing its spectral data with the literature.<sup>29</sup>

**Oleic acid (C 3):** Pale yellow, [M]<sup>+</sup>  $m/z$  283, molecular formula C<sub>18</sub>H<sub>34</sub>O<sub>2</sub>. UV:  $\lambda$  max 317 nm. <sup>1</sup>HNMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  2.36 [t,  $J = 7.5$  Hz, H-2], 1.63, 1.32 [m, H-3,7] respectively, 1.27-1.34 (20H), 2.02 [m, H-8,11], 5.4 [m, H-9,10], 0.8 (t,  $J = 7.0$  Hz, H-18), 11.26 (OH). <sup>13</sup>C NMR [125 MHz: (CDCl<sub>3</sub>):  $\delta$  33.80, 24.96, 29.50, 29.51, 29.44, 30.10, 27.25 [C(2,3,4,5,6,7,8)] respectively, 180.65 (C-1), 129.84, 130.50

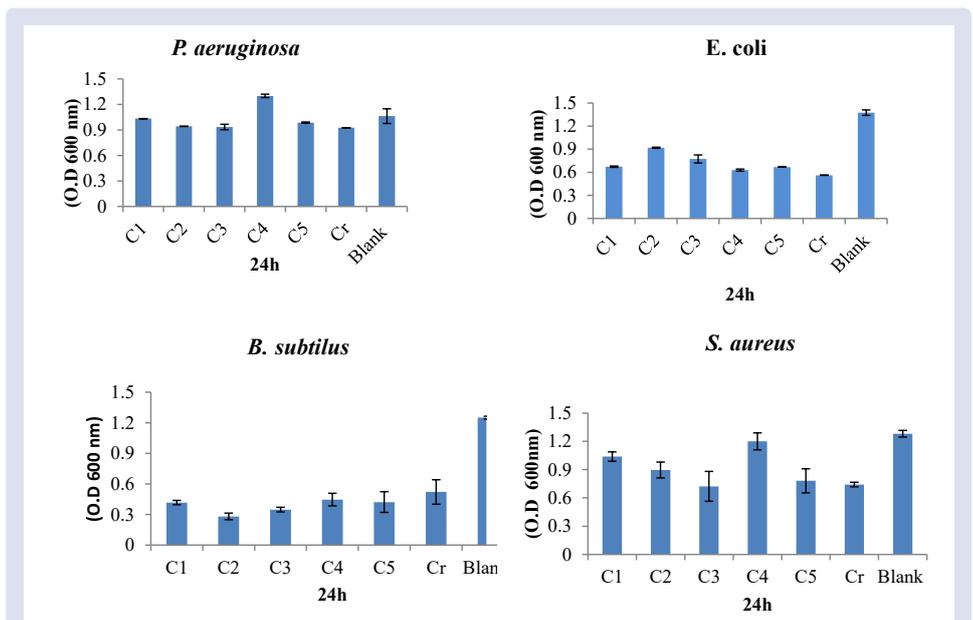
[C(9,10)] respectively, 27.52, 29.60, 29.50, 29.43, 29.25, 31.94, 22.74 [C(11,12,13,14,15,16,17)] respectively, 14.40 (C-18). Comparison of the spectral data with literature confirmed compound.<sup>30</sup>

**Nicotinamide (C 4):** Colorless, [M]<sup>+</sup>  $m/z$  123, molecular formula C<sub>6</sub>H<sub>6</sub>N<sub>2</sub>O. UV:  $\lambda$  max 261 nm. <sup>1</sup>HNMR (500 MHz, DMSO-d<sub>6</sub>):  $\delta$  7.54 (dd,  $J = 7.9, 8.0$  Hz, H-5), 8.24, 8.68 [dd, H-4,6,  $J = 7.9, 1.6$  Hz] respectively, 8.91 (s, H-2). <sup>13</sup>C NMR [125 MHz: DMSO-d<sub>6</sub>):  $\delta$  152.5, 131.4, 137.1, 124.9, 149.5 [C(2,3,4,5,6)] respectively, 170.6 (C-1). Comparison of the spectral data with literature confirmed compound.<sup>31</sup>

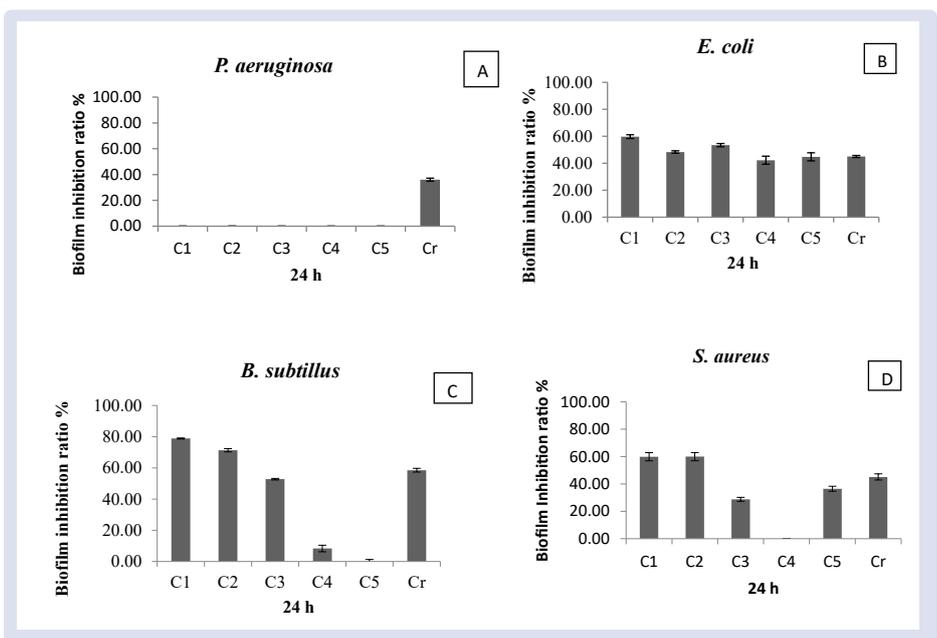




**Figure 4:** GC/MS chromatogram of the compounds identified in ethyl acetate extract of cutler filtrate of *Sarcocladium kiliense* SDA20.



**Figure 5:** Antibacterial activity of crude and pure compounds of *Sarcocladium kiliense* SDA20 against pathogenic bacterial strains.



**Figure 6:** Biofilm inhibitory ratio % following pre-treatment with Crude and pure compounds. N.B. A: *P. aeruginosa*, B: *E. coli* and, C: *B. subtilis* & D: *S. aureus*. Data presented represents mean  $\pm$  SD of three independent experiments. Grey columns representative of pre-treated samples.

**Table 3: Compounds identified in ethyl acetate extract of cutler filtrate of *Sarocladium kiliense* SDA20 by GC-MS.**

Peak	Compound Name	Molecular Formula	RT	Area %
1	Thiophene, 2,5-diethyl-	C8H12S	13.444	1.37
2	Propanedioic acid, phenyl-	C9H8O4	14.068	1.68
3	Undecanoic acid	C11H22O2	19.263	0.97
4	2-Phenyl-1-p-toluenesulfonylaziridine	C15H15NO2S	25.643	1.34
5	Erucic acid	C22H42O2	26.341	0.63
6	2-Hexadecanol	C16H34O	26.559	1.09
7	Cyclopropanedodecanoic acid, 2-octyl-, methyl ester	C24H46O2	27.08	1.45
8	cis-13-Eicosenoic acid	C20H38O2	27.772	0.72
9	Phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl-	C9H9F3O2	27.995	0.5
10	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	C17H24O3	29.019	1.25
11	Hexadecanoic acid, methyl ester	C17H34O2	29.128	15.24
12	Heptacosane	C27H56	30.518	2.36
13	Hexadecanoic acid, 14-methyl-, methyl ester	C18H36O2	31.039	0.58
14	2-Hexadecanol	C16H34O	31.131	1.65
15	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C19H34O2	32.292	8.5
16	9-Octadecenoic acid (Z)-, methyl ester	C19H36O2	32.418	22.26
17	11-Octadecenoic acid, methyl ester	C19H36O2	32.504	2.44
18	Octadecanoic acid, methyl ester	C19H38O2	32.876	3.48
19	Estra-1,3,5(10)-trien-17.β.-ol	C18H24O	34.141	2.11
20	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	C26H54	37.465	1.44
21	Oleic acid, eicosyl ester	C38H74O2	38.226	0.48
22	Oleic acid, 3-(octadecyloxy)propyl ester	C39H76O3	39.039	0.44
23	Diisooctyl phthalate	C24H38O4	39.805	10.76
24	unidentified	-----	40.549	0.79
25	4,6,8(14)-Cholestatriene	C27H42	44.423	0.49
26	Cholest-5-en-3-ol	C27H46O	47.61	11.15
27	unidentified	-----	54.517	4.86

**Table 4: Minimal Inhibitory Concentrations (MICs) of crude extract and pure compounds (µg/mL).**

Compound no. M.O. used	Minimum inhibitory concentration (MIC)			
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
Cr	1	1	-	1
C1	-	2	-	1
C2	1	3	-	2
C3	1	2	-	1
C4	-	2	-	1
C5	1	2	-	1

N.B.: 1 = 250 µg/mL; 2 = 125 µg/mL; 3 = 62.5 µg/mL; -: no result.

preliminary antibiofilm results of the crude extract displayed moderate biofilm inhibition activity against *B. subtilis*, *S. aureus* and *E. coli* with biofilm inhibitory ratio of 58, 45 and 45% respectively, and low biofilm inhibition activity against *P. aeruginosa* up to 36%. The pure compounds were also evaluated for their ability to eradicate bacterial biofilm formation. As a result, C 1, C 2 reduced the biofilm formation of the strain *B. subtilis* up to 78 % and 71 % respectively, followed by C 3 (52%) (Figure 6A). *S. aureus* biofilm formation was inhibited over 59 % by C 1 and C 2, while C 3 and C 5 have a low inhibition activity (Figure 6D). In case of *E. coli*, the highest percentage of biofilm inhibition was shown by C1 (59%) and C 3 (53%) followed by C 2, C 5 and C 4 (Figure 6B). On the other hand, *P. aeruginosa* strain didn't display any response to isolated pure compounds (C 1: C 5) (Figure 6C). Recently, many researchers have been studied the relationship between biofilm formation and the persistence of bacterial infections. They reported that, once bacteria form a biofilm, they become more resistant to antibiotic.<sup>15</sup> For this reason, bacterial biofilms are considered as a remarkable source of pathogenic diseases such as osteomyelitis, dental caries, otitis media and nosocomial infections such as urinary tract, lower respiratory tract and surgical site infections.<sup>16,17,36,37</sup> In this context, many trials have been performed to find an effective antibiofilm agents. In the study

conducted by<sup>38</sup> the extract of endophytes *Chlamydomonas* sp. was found to be very potential as antibiofilm agent against *P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145.<sup>38</sup>

## DISCUSSION

Bioactive compounds were isolated from culture filtrate of *Sarocladium kiliense* SDA20 grown in fermentation media containing shrimp shell waste as substrate. This media produced the high activity in the mycotoxins production under optimum conditions using inoculum size 10<sup>6</sup> spores/ml of *Sarocladium kiliense* SDA20 at pH 7.0, as shown in (Table 2) and (Figure 2). The ethyl acetate extract of culture filtrate of *Sarocladium kiliense* SDA20 was subjected to silica gel column chromatography, The fractions obtained were examined using TLC and the similar fractions were collected to give five major fractions, from which 5 compounds were isolated for the first time, Cholest-5-en-3-ol, palmitic acid, oleic acid, nicotinamide and tricin The isolated compounds were identified by spectroscopic methods and comparing data with reported literature. GC-MS analysis of ethyl acetate extract of culture filtrate showed the presence of twenty-seven phytochemical compounds. The identification of compounds was based on comparing

the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data. The crude extract of the *Sarcocladium kiliense* SDA20 and the five isolated pure compounds were tested against four pathogenic bacterial strains. The crude extract showed antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*, while no activity has been detected toward *Pseudomonas aeruginosa*. All pure compounds displayed a strong inhibitory activity against *Bacillus subtilis*. On the other hand, Compounds (C1, C4 and C5) showed moderate antibacterial activity against *E. coli*, followed by compound (C2 and C3). Meanwhile *S. aureus* was less susceptible to pure compounds with very low activity of compounds (C3 and C5). Using MTT assays, the biofilm inhibition activity of the crude extract and isolated pure compounds were assayed toward four pathogenic bacteria (*P. aeruginosa*, *S. aureus*, *E. coli* and *B. subtilis*). Results revealed that the crude extract and isolated pure compounds displayed moderate biofilm inhibition activity against the four pathogenic bacteria.

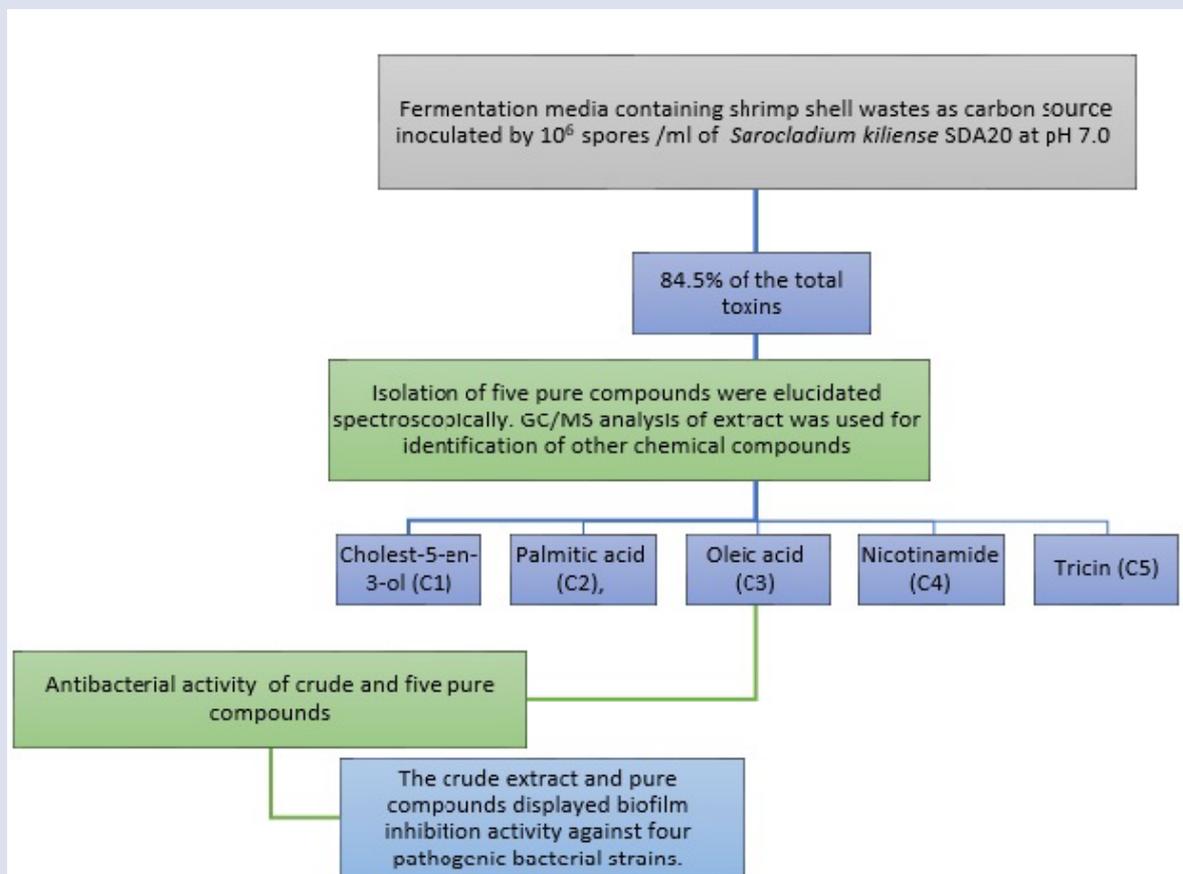
## CONCLUSION

Five compounds were isolated for the first time from the ethyl acetate extract of culture filtrate of *S. kiliense* MN636431. Also, twenty-seven compounds from GC-MS analysis of the extract were identified. Tested the antibacterial activity and MTT assay of crude and five pure compounds displayed a strong inhibitory antibacterial activity against different bacterial strains and the biofilm inhibition activity. The use of fungal endophytes in drug discovery have many advantages due to their diversity that offers an abundant source of active and novel metabolites. On the other hand, the utilization of fungal endophytes can be the act as an alternative source of bioactive metabolites extracted from plants that lead to eliminating of large quantity of plant material.<sup>39</sup>

## REFERENCES

- Schollenberger M, Muller HM, Ruffe M, Terry-Jara H, Suchy S, Plank S, et al. Natural occurrence of *Fusarium* toxins in soy food marketed in Germany. *Int J Food Microbiol*. 2007;113:142-6.
- Ghali R, Hmaissia-khlifa K, Ghorbel H, Maaroufi K, Hedili A. Incidence of aflatoxins, ochratoxin A and zearalenone in tunisian foods. *Food Control*. 2008;19:921-4.
- Osman MO El – M, Yassmin MS. Detection of mycotoxin produce by endophytic fungi. *IJLSR*. 2014;2(4):37-42.
- Rodrigo MC, Maria MGP, Amilton PRC, Adriana MT, Maria CSM. Toxicogenic mycobiota and mycotoxins in shrimp feed. *Ciênc Rural*. 2015.
- Selim KA, El-Beih AA, Abdel-Rahman TM, El-Diwany AI. Biology of endophytic fungi. *Curr Res Environm Appl Mycol*. 2012;2(1):31-82.
- Keller NP, Turner G, Bennett JW. Fungal secondary metabolism - from biochemistry to genomics. *Nat Rev Microbiol*. 2005;3(12):937-47.
- Adejoye OD, Adebayo-Tayo BC, Ogunjobi AA, Olaoye OA, Fadahunsi FI. Effect of carbon, nitrogen and mineral sources on growth of pleurotus Florida, a Nigeria edible mushroom. *Afr J Biotechnol*. 2006;5:1355-9.
- Andualem B, Gessesse A. Production of microbial medium from defatted brebra (*Milletia ferruginea*) seed flour to substitute commercial peptone agar. *Asian Pac J Trop Biomed*. 2013;3:790-7.
- Demirbas A. Political, economic and environmental impacts of biofuels: A review. *Appl Energy*. 2009;86:S108-17.
- Gillett RD. Global study of shrimp fisheries. *FAO DocTech Pêches*. 2008;475:331.
- Atalla SMM, Gamal NGEL, Awad HM. Chitinase of marine *Penicillium chrysogenum* MH745129: Isolation, identification, production and characterization as controller for citrus fruits postharvest pathogens. *JJBS*. 2019.
- Chapman MR, Robinson LS, Pinkner JS, Roth R, Heuser J, Hammar M, et al. Role of *Escherichia coli* curl operons in directing amyloid fiber formation. *Science*. 2002;295:851-5.
- Branda SS, Vik S, Friedman L, Kolter R. Biofilms: The matrix revisited. *Trends Microbiol*. 2005;13:20-6.
- Steinberg N, Kolodkin-Gal I. The matrix reloaded: Probing the extracellular matrix synchronizes bacterial communities. *J Bacteriol*. 2015;197:2092-103.
- Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO. Biofilm formation mechanisms and targets for developing antibiofilm agents. *Future Med Chem* 2015;7:493-512.
- Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev*. 2002;15:167-93.
- Wingender J, Flemming HC. Biofilms in drinking water and their role as reservoir for pathogens. *Int J Hyg Environ Health*. 2011;214:417-23.
- Ingebrigtsen RA, Hansen E, Andersen JH, Eilertsen HC. Light and temperature effects on bioactivity in diatoms. *J Appl Phycol* 2016;28:939-50.
- Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother*. 2001;48(suppl. 1):5-16.
- Antunes AL, Trentin DS, Bonfanti JW, Pinto CC, Perez LR, Macedo AJ, Barth AL. Application of a feasible method for determination of biofilm antimicrobial susceptibility in staphylococci. *APMIS*. 2010;118(11):873-7.
- Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *MBE*. 2016;33:1870-4.
- Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Boil Evol*. 1978;4:406-25.
- Hams MAM, Walaa FA E, Albert B, Seddik H. Detection of aflatoxin producing fungi isolated from Nile tilapia and fish feed. *Excli J*. 2017;16:1308-18.
- Obire O, Okigbo RN, Minimah T R. Mycoflora of fresh shrimps (*Penaeus aztecus*) from different markets in Port Harcourt, Nigeria. *Afri J Food Sci*. 2013;7(8):204-9.
- Miller DJ. Screening for secondary metabolites. *Fungal Divers Press*. 2017;158-71.
- Pikuta EV, Marsic D, Itoh T, Bej AK, Tang J, Whitman WB, Garrlott OK, Hoover RB. *Thermococcus thioreducens* sp. nov., a novel hyperthermophilic, obligately sulfur-reducing archaeon from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol*. 2007;57:1612-8.
- Reysenbach AL, Liu Y, Banta AB, Beveridge TJ, Kirshtein JD, Schouten S, et al. A ubiquitous *thermoacidophilic* archaeon from deep-sea hydrothermal vents. *Nature*. 2006;442:444-7.
- Adane L, Tesemma M, Tariku Y, Muleta D, Demise S. Isolation of compounds from acetone extract of root wood of *Moringa stenopetala* and evaluation of their antibacterial activities. *Res J Med Plant*. 2013;7(1):32-47.
- Eskander DM, El-Khrisy EDA, Grace MH, Nabil M, Nassar MI, Mounier MM. Investigation of secondary metabolites and cytotoxicity of *Jacquemontia pentantha* (Jacq.). *Phcog J*. 2019;11(4):712-7.
- Yamada S, Ab M, Ito Y, Suzuki A, Onoue S, Noguchi H. Isolation and pharmacological characterization of fatty acids from Saw Palmetto extract. *Anal Sci*. 2009;25:553-7.
- Yamashita N, Sakata K, Ina H, Ina K. Isolation of nicotinamide from *Mallotus* leaves as an attaching repellent against the blue mussel, *Mytilus edulis*. *Agric Biol Chem*. 1989;53(12):3351-2.
- Ho WS, Wang Y, Shen JZ, Chan YW. Identification and growth inhibitory activity of the chemical constituents from *Imperata cylindrica* aerial part ethyl acetate extract. *Molecules*. 2018;23(7):1807.
- Pimentel MR, Molina G, Dionísio AP, Maróstica Junior MR, Pastore GM. The use of endophytes to obtain bioactive compounds and their application in biotransformation process. *Biotechnol Res Int*. 2011;11.
- Yang HR, Hu XP, Jiang CJ, Qi J, Wu YC, Li W, et al. Diversity and antimicrobial activity of endophytic fungi isolated from *Cephalotaxus hainanensis* Li, a well-known medicinal plant in China. *Lett Appl Microbiol*. 2015;61:484-90.
- Gashgari R, Gherbawy Y, Ameen F, Alsharari S. Molecular characterization and analysis of antimicrobial activity of *Endophytic Fungi* from medicinal plants in Saudi Arabia. *JJM*. 2016;9(1):e26157.
- Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science*. 1999;284:1318-22.
- Miquel S, Lagrèfeuille R, Souweine B, Forestier C. Anti-biofilm activity as a health issue. *Front Microbiol*. 2016;7:592.
- Nithya C, Lewisoscar F, Kanaga S, Kavitha R, Bakkiyaraj D. Biofilm inhibitory potential of *Chlamydomonas* sp. extract against *Pseudomonas aeruginosa*. *JABU*. 2014;5(4):74-81.
- Strobel GA, Hess WM, Ford E, Sidhu RS, Yang X. Taxol from fungal endophytes and the issue of biodiversity. *J Ind Microbiol*. 1996;17(5):417-23.

## GRAPHICAL ABSTRACT



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